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Time Course of Restenosis During the First Year After Emergency Coronary Stenting

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Background. **Prevention of abrupt vessel closure after percutaneous transluminal coronary angioplasty (PTCA) represents one of the current indications for intracoronary stent implantation. After the procedure, the stented segment undergoes luminal changes that may lead to late restenosis. This study was undertaken to assess the time course of luminal changes during the first year after emergency placement of coronary stents.**

Methods and Results. **Coronary stenting was indicated in patients with present or threatened vessel closure secondary to large dissections after PTCA. From June 1989 to May 1991, 82 patients who received Palmaz-Schatz stents and did not have early vessel occlusion after stenting were enrolled into a serial angiographic follow-up study. Coronary normal reference diameter and minimal luminal diameter were measured with an automated edge detection technique. Patients who underwent repeat PTCA for restenosis were excluded from further serial angiography. The restudy rate at 3, 6, and 12 months was 96%, 81%, and 90% of the eligible patients, respectively. The incidence of restenosis (defined as a diameter stenosis ≥50%) was 22.0% at 3 months, 31.9% at 6 months, and 33.2% at 12 months. Minimal luminal diameter was increased from 0.66±0.32 mm before to 2.85±0.43 mm immediately after stenting. It was 0.46±0.31 mm smaller than the diameter of the maximally inflated balloon during the procedure. The reduction in minimal luminal diameter was 0.80±0.69 mm (p=0.0001) for the first 3 months, 0.29±0.52 mm (p=0.0001) between 3 and 6 months, and 0.13±0.32 mm (p=0.01) for the last 6 months. The percentage of patients who presented a significant change in minimal luminal diameter (defined as >0.60 mm) declined from 50.6% during the first 3 months and 18.9% between 3 and 6 months to 6.5% for the period between 6 and 12 months.**

Conclusions. **The incidence and the time course of restenosis after emergency coronary stenting are similar to that reported for conventional PTCA. Coronary lumen dimensions demonstrated a peak change at 3 months and remained mostly stable after the first 6 months. (*Circulation* 1993;87:1498–1505)**

KEY WORDS • **stents** • **coronary angioplasty** • **coronary dissection** • **coronary occlusion**

Coronary stenting is currently used for circumventing two major limitations of percutaneous transluminal coronary angioplasty (PTCA): early acute occlusion and restenosis of the dilated vessel.[1,2]

Abrupt vessel closure after PTCA occurs in 2–9% of the cases.[3–7] It is often associated with acute ischemic complications and/or the need of emergency aortocoronary bypass surgery.[7,8–10] Extensive coronary dissection of the dilated vessel has been found to be the most frequent precursor of this complication.[6,11]

Restenosis involves one third of the patients undergoing PTCA.[12] The process of restenosis seems to begin as early as immediately after the procedure. Elastic recoil, vasospasm, platelet aggregation, and thrombus formation are factors that alone or in combination contribute to the early luminal diameter reduction of the dilated vessel.[12,13] Smooth muscle cell proliferation, migration, and elaboration of extracellular matrix later become the dominant mechanisms of the restenosis.[13,14] Two well-designed angiographic studies have found that most of the narrowing of the dilated coronary arteries occurs within the first 3 months, and only minimal changes develop after 6 months.[15,16] Accordingly, the process of restenosis after PTCA seems to be a time-limited phenomenon, and the 6-month restenosis rate has been accepted to represent the definitive incidence of this complication.

Implantation of coronary stents is also associated with the occurrence of early occlusion secondary to stent thrombosis and late restenosis.[17–19] Experimental animal studies[20] and pathological data in humans[21,22] have shown that in addition to the mechanisms acting after PTCA, the interactions between stent and vessel wall affect the phenomenon of restenosis after the procedure. The presence of a foreign body in the vascular wall may influence the time course of restenosis in a way that is still unclear.[22] Observations made in a porcine model demonstrate that progression of intimal hyperplasia after stenting continues for a much longer period than after balloon injury alone.[23] Yet, the poten-

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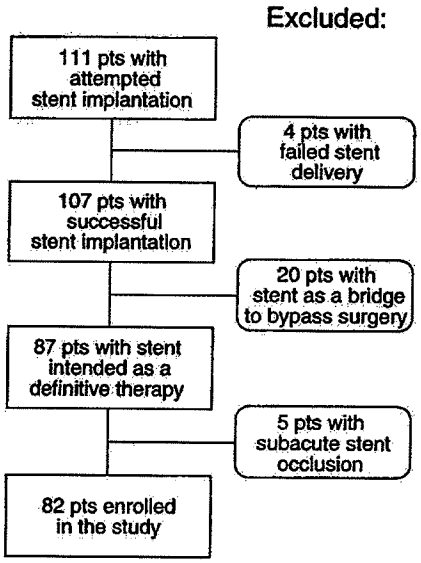

FIGURE 1. *Chart of patients (pts) excluded from the angiographic follow-up study.*

tial influence of the atherosclerotic plaque on the response to injury is usually absent in animal studies, and great care must be taken in extrapolating these findings to humans.[24] The knowledge of the temporal sequence of restenosis after coronary stenting is important not only for scheduling the follow-up of the patients but also for planning possible preventive measures. This study was designed to assess the time course of luminal changes after emergency coronary stenting by means of serial quantitative angiography.

Methods

Patients

During a 24-month period from June 1989 to May 1991, emergency placement of a Palmaz-Schatz stent (Johnson & Johnson) was attempted in 111 of 2,052 patients submitted to PTCA. The indication for emergency stent implantation was extensive coronary dissection during PTCA with present or threatened vessel closure despite multiple balloon dilations in a patient without major contraindications to anticoagulation therapy. All patients gave written informed consent before the intervention.

Coronary stents were successfully delivered in 107 (96%) of the patients (Figure 1). Twenty patients belonging to our early experience underwent elective bypass surgery within 1 week from the procedure in accordance with our initial policy to use stent implantation as a bridge to surgical intervention. The target lesion in most of these patients was situated proximally in the left anterior descending coronary artery, and no clinical signs of stent occlusion were observed up to the day of the bypass operation. Because of the short time delay, they had no angiographic control before surgery and were excluded from the follow-up protocol of the present study. Five additional patients who had clinical and angiographic evidence of early thrombotic stent occlusion within the first 2 weeks after the procedure were also excluded. The remaining 82 patients were enrolled into a prospective follow-up study including serial angiographic control at 3, 6, and 12 months. Clinical events were recorded, and patients presenting with recurrent anginal symptoms were recatheterized before the preset time. Baseline characteristics of the patients are listed in Table 1.

TABLE 1. **Main Characteristics of the Study Patients**

No. of patients	82
Sex (M/F)	65/17
Age (years)	58.9±9.3
Angina pectoris	
Unstable	35
Stable	47
Active smokers	17
Hypercholesterolemia	41
Systemic hypertension	46
Diabetes mellitus	15
Single-vessel disease	39
Multivessel disease	43
Prior PTCA	27
Coronary dissection associated with total occlusion before stenting	10
Stent localization	
LAD	19
LCx	16
RCA	44
Bypass graft	3
Single-stent patients	77
Multiple-stent patients	5

PTCA, percutaneous transluminal coronary angioplasty; LAD, left anterior descending coronary artery; LCx, left circumflex artery; RCA, right coronary artery.

Stent implantation was performed using the technique described previously.[18] Anticoagulation was instituted according to the following regimen: 1) 15,000 units of heparin and 500 mg of aspirin were administered intravenously during the procedure. 2) Immediately after the procedure, an oral vitamin K antagonist (Marcumar, Hoffmann-La Roche AG) and aspirin (100 mg per day) were started. Heparin infusion was adjusted according to the activated partial thromboplastin time and continued until stabilization of the therapeutic level of oral anticoagulation (generally for 7–10 days). Patients were usually discharged after 2 weeks. Oral anticoagulation was discontinued 2 months after the procedure.

Quantitative Coronary Angiography and Definitions

Coronary arterial dimensions were assessed in a single matched "worst view." The same projection that showed the most severe stenosis before the intervention was used throughout the study. The selected cineframe was digitized with a spatial resolution of 1,850 dots per inch and 256 gray levels by means of a ScanMaker 1850S slide scanner (Microtek International, Inc., Taiwan) connected to a Macintosh Quadra 900 computer (Apple Computer, Inc.). The edge detection software was developed in our laboratory and based on the weighted sum of the first and second derivative applied to the digitized brightness information.[25] The contour was automatically detected after choosing the region of

interest and an approximate centerline of the arterial segment containing the stenosis. The same software was used for measuring the actual balloon size used during stent placement. The absolute dimensions of the vessel and the diameter of the final stent expansion balloon were computed using the guiding catheter as a scaling device. Percent diameter stenosis was calculated using minimal luminal diameter and interpolated reference diameter.[25] In a few patients in whom opacification distal to the stenosis was too poor to allow normal contour detection, the diameter of the normal segment proximal to the stenosis was used as normal reference diameter. Because of the poor visibility of Palmaz-Schatz stents, no attempts were made to distinguish whether restenosis lay within or in the proximal or distal segments adjacent to the stent. The quantitative assessment was carried out on frames before PTCA, immediately after stent placement, and at each follow-up angiographic control. There were no measurements made immediately after PTCA because the presence of large dissections in our patients (as per indication) would preclude any exact analysis. The difference, if any, between the diameter of the final stent expansion balloon and the resulting minimal luminal diameter was considered to be caused by elastic recoil of the stented coronary segment.

The variability of coronary measurements inherent to our method was assessed in 23 stented patients who had angiograms with matching projections performed at our institution at two different times (diagnostic and immediately preceding PTCA). The size of the catheters used for most of the analyzed angiograms was 6F for the diagnostic and 8F for the intervention session. The median interval between the two angiographies was 21 days. The difference (mean±SD) between the two measurements was 0.06±0.30 mm for minimal luminal diameter and 0.09±0.28 mm for the normal reference diameter. Any change in minimal luminal diameter more than double of the standard deviation (0.60 mm) during follow-up studies was considered as significant. Restenosis was defined as a percent diameter stenosis ≥50% found at control angiography.

Angiographic Follow-up

As shown in Figure 2, 79 patients (96%) had angiography at 3 months (median, 100 days); 13 patients with restenosis who had repeat PTCA were excluded from further follow-up. Six-month angiography (median, 192 days) was performed on 56 of the remaining 69 patients (81%), and six other patients were submitted to PTCA. Fifty-seven of the 63 patients without prior PTCA (90%) had angiographic study at 12 months (median, 368 days). All 82 patients had at least one control angiography, and the reason for missing studies was always patient refusal. The three patients with missing angiographic study at 3 months had angiography at both 6 and 12 months (except one who underwent repeat PTCA at 6 months). Eleven of the 13 patients without angiography at 6 months and four of the six patients with no angiography at 12 months had already completed two of the three required controls.

Statistical Analysis

Values are expressed as mean±SD. Restenosis rate was calculated according to the actuarial method and

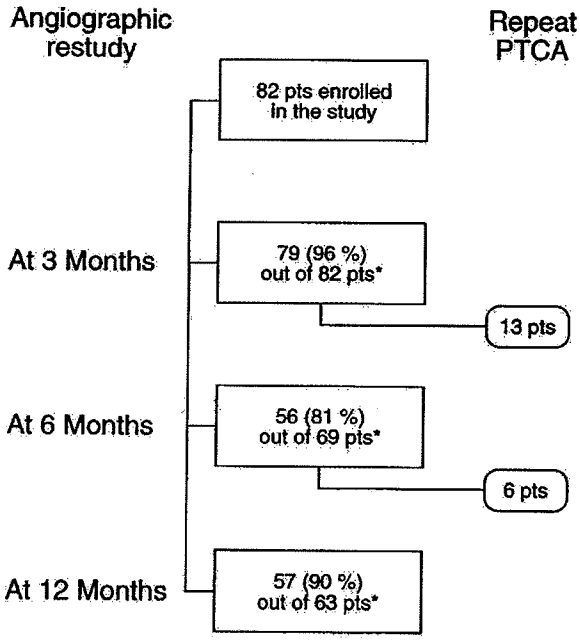

FIGURE 2. *Chart of angiographic follow-up of the 82 stented patients (pts) included in the study. *The number of the eligible patients at the given follow-up period is equal to the total number (82 patients) minus number of patients who had previously undergone repeat percutaneous transluminal coronary angioplasty (PTCA) after stent implantation.*

expressed in percent±1.96 SEM. Two-tailed *t* test and ANOVA followed by Scheffe's test were used to check for significant differences between the means of continuous variables. Values of $p<0.05$ were considered significant.

Results

Before the intervention, the patients presented a coronary normal reference diameter of 3.26±0.64 mm, a minimal luminal diameter of 0.66±0.32 mm, and a diameter stenosis of 79.5±9.6%. The size of the balloon used for stent deployment was 3.31±0.38 mm.

Coronary stenting was associated with an increase in minimal luminal diameter from 0.66±0.32 mm to 2.85±0.43 mm and a marked reduction in percent diameter stenosis from 79.5±9.6% to 17.4±6.9%. Normal reference diameter did not change during the study period (3.25±0.5 mm at 1-year control versus 3.26±0.64 mm before stent implantation).

Cases of death or myocardial infarction were not observed during the study period. Figure 3 illustrates the time course of restenosis during the entire period of the study. Restenosis rate was 22.0±7.9% at 3 months. It increased to 31.9±9.5% at 6 months and showed only a minimal further rise to 33.2±10.4% at 12 months (Figure 3). The cumulative number of patients with restenosis was 18, 26, and 27 at 3, 6, and 12 months, respectively. During the study period, five of the 10 patients with vessel wall dissection and total occlusion after PTCA had restenosis after stenting. Fifteen of the 27 patients with angiographic restenosis had recurrent angina pectoris during follow-up.

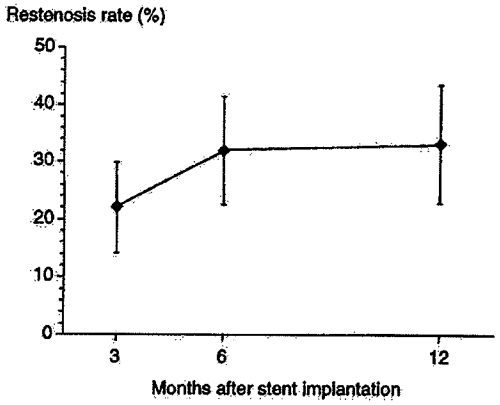

FIGURE 3. *Graph of restenosis rate at the three angiographic studies. Error bars represent ±1.96 SEM.*

The difference between diameter of the final stent expansion balloon and minimal luminal diameter immediately after stent placement was 0.46±0.31 mm (Figure 4); 14% of the lumen diameter was lost because of elastic recoil of the stented segment.

Minimal luminal diameter demonstrated a change of −0.80±0.69 mm from immediately after stenting to 3 months (p=0.0001); during the same interval, percent diameter stenosis was augmented by 19.6±20.2% (p=0.0001). Seven patients met the criteria of restenosis after 6 months but did not do so at 3 months. These patients, however, tended to have more reduction in minimal luminal diameter during the first 3 months than the other 42 patients without restenosis in both studies (−0.78±0.42 mm versus −0.46±0.44 mm, p=0.08). From 3 to 6 months, the change in minimal luminal diameter was −0.29±0.52 mm (p=0.0001), and the change in percent diameter stenosis was 7.8±12.9% (p=0.0001). The changes were much lower between 6 and 12 months: −0.13±0.32 mm for minimal luminal diameter (p=0.01) and 3.2±9.5% for percent diameter stenosis (p<0.05). Five patients with restenosis at 6 months who did not require repeat PTCA showed a change in minimal luminal diameter between 6 and 12 months comparable to that of patients without restenosis (0.08±0.28 mm versus −0.16±0.32 mm, p=NS).

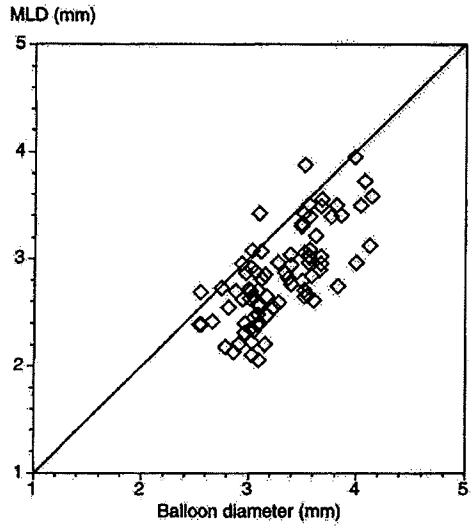

FIGURE 4. *Scatterplot of relation between the diameter of the maximally inflated balloon during stent placement procedure and the resulting coronary minimal luminal diameter (MLD). Solid line represents the line of identity.*

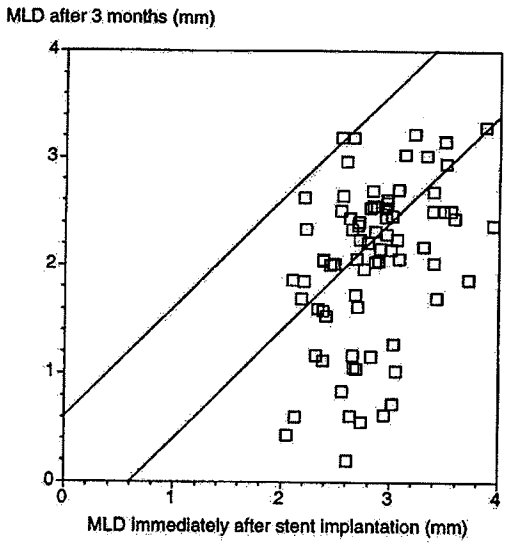

FIGURE 5. *Scatterplot of progression of coronary luminal narrowing between immediately after and 3 months after stent implantation in 79 patients with both angiographic controls. MLD, minimal luminal diameter. Solid lines represent twice the variability (0.60 mm) for repeated measurements of MLD of the same lesion during two separate angiographic studies.*

The proportion of patients who demonstrated a significant lumen change (>0.60 mm) between two consecutive angiographic studies was decreasing from 3 to 12 months. From immediately after stenting to 3 months, 40 patients (50.6%) presented a reduction in minimal luminal diameter >0.60 mm (Figure 5). The same criterion was fulfilled by 10 patients (18.9%) between 3 and 6 months (Figure 6) and only by three patients (6.5%) from 6 to 12 months (Figure 7).

Discussion

Coronary stenting is one of the recent therapeutic and device-aided interventions aimed at reducing the incidence of abrupt vessel closure and late restenosis after PTCA.[1] Restenosis after PTCA is a complex process not completely understood. It is thought, however, that lesion and regional flow characteristics are both major determinants of the degree of restenosis.[13] The improvement of coronary stenosis geometry and flow characteristics with stents[26] seems to be useful for preventing restenosis after PTCA. In addition to the improvement of flow characteristics, stents have been used successfully to tack up intimal and medial flaps.[1] Both of these features have been the rationale for the management of patients with acute coronary occlusion after PTCA since 1986.[27]

We resorted to emergency stent implantation in 5% of the total number of patients undergoing PTCA. All of them had either complete or impending vessel closure secondary to large wall dissections. The 5% rate in our

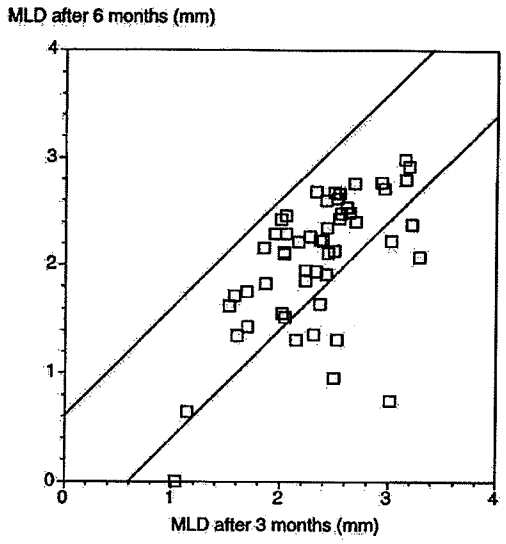

FIGURE 6. *Scatterplot of progression of coronary luminal narrowing between 3 and 6 months after stent implantation in 53 patients with both angiographic controls. MLD, minimal luminal diameter. Solid lines represent twice the variability (0.60 mm) for repeated measurements of MLD of the same lesion during two separate angiographic studies.*

series does not differ from the known incidence of acute complications after PTCA.[3-7]

Methodological Aspects

Although coronary angiography is unable to elucidate the mechanisms responsible for the restenosis, it remains the best tool for evaluating its degree, incidence, and time course after diverse coronary interventions.[28,29]

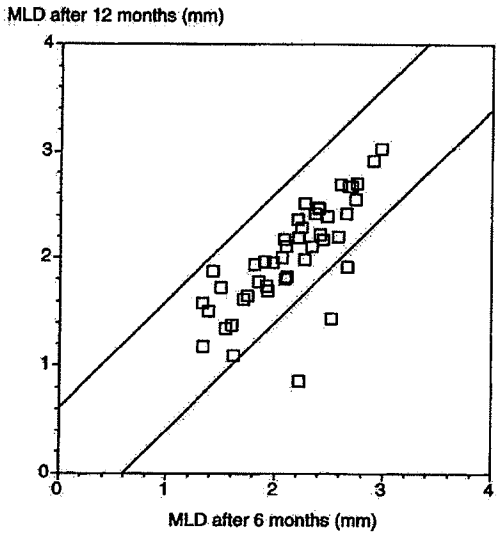

FIGURE 7. *Scatterplot of progression of coronary luminal narrowing between 6 and 12 months after stent implantation in 46 patients with both angiographic controls. MLD, minimal luminal diameter. Solid lines represent twice the variability (0.60 mm) for repeated measurements of MLD of the same lesion during two separate angiographic studies.*

We used serial angiographic follow-up to assess the time course of luminal changes after emergency coronary stent implantation. The high angiographic restudy rate achieved at all three preset times in our patients avoids the selection bias that usually arises if only symptomatic patients are controlled. The assessment of coronary luminal changes was done by means of a quantitative angiographic system. Because of the proven superiority of the automated quantitative methods,[30] they have become a widely accepted requirement for reporting the restenosis rate.[12,31]

The use of a single worst view to measure the coronary arterial dimensions in the present study may be arguable. A recent comparative study has found that coronary stenosis measurements made in the single worst view correlate very well with those obtained using two orthogonal views.[32] Coronary stenting is known to remodel the stented segment into a more circular configuration,[33] and further improvement of the reliability of single-view measurements is to be expected during follow-up studies.

The serial evaluation of coronary angiograms was preceded by a variability study performed in 23 patients. The difference in minimal luminal diameter obtained with a paired analysis of two separate coronary cineangiograms at a median of 21 days apart showed a standard deviation of 0.30 mm. This value differs somewhat from that of 0.36 mm reported by Reiber et al[34] and of 0.25 mm found in the study of Nobuyoshi et al[16] in a comparable number of patients. The time interval between the two angiograms was 90 days for the former and 9 days for the latter study. Differences in the time delay and those in the hardware and software features of the system used for the measurements may account for the differences in the variability limit obtained in these studies. We used the 0.60 mm value to distinguish the significant changes in coronary lumen between two consecutive angiographies because our main objective was the evaluation of serial changes. On the other hand, the presence of patients with repeat PTCA makes any further analysis difficult.

General consensus about the angiographic definitions of restenosis is lacking.[28] To achieve comparability with other studies, we chose the most frequently used definition, that of a percent diameter stenosis ≥50%. Recently, the absolute change in luminal diameter was proposed as a more reliable index of restenosis.[15] Definitions based on absolute changes reflect hyperplasia as an important component in the process leading to restenosis. Restenosis is, however, a wider concept that includes the initial result as well. This is particularly relevant in the case of stenting that achieves a larger lumen than PTCA alone. Coronary stenosis, on the other hand, and consequently all the interventional decisions taken in the clinical practice, are based on percent diameter stenosis. It is desirable to apply for restenosis the definitions that are similar to the one that led to the intervention.

Elastic Recoil

We assessed the elastic recoil phenomenon during the stent placement procedure. Whether the recoil process is extended beyond the time of the intervention is still unclear. Controls performed the day after PTCA yielded conflicting results in this regard. Nobuyoshi et

al[16] found a significant decrease in minimal luminal diameter in 185 patients from immediately after PTCA to day 1, whereas one other study reported no significant difference at the same interval in 16 patients.[35] Coronary arterial dimensions appeared to be stable during the first 24 hours after Palmaz-Schatz stent placement.[18,36]

The vascular recoil of 14% found in this study is similar to that of 15% reported after stent placement in 167 patients[37] and higher than that of 3% found by de Jaegere et al[38] in 50 patients who received the Wiktor stent. The difference, however, is not explained by different scaffolding properties of the stents that were used. The more plausible explanation is the different method used for the calculation of elastic recoil. de Jaegere et al[38] used the mean diameter of the stented segment that is obviously greater than minimal luminal diameter used by us. If the same method is applied for their data, the difference between the diameter of the inflated balloon and the minimal luminal diameter is 0.53 mm, similar to that observed in the present study. The vascular recoil for the same Wiktor stent was 9% when implanted in the normal coronary arteries of Yorkshire pigs.[39] We cannot exclude that for stent implantation, other mechanisms than for PTCA may be responsible for the immediate loss in lumen diameter after deflation of the balloon–stent assembly. Angioscopy has shown that stenotic segments bulge into the lumen of human coronary arteries at the articulation site of Palmaz-Schatz stents.[40] This mechanism may have been even more operative in our patients who had important vessel wall disruption secondary to large dissections. Nevertheless, Palmaz-Schatz stenting in the present study was associated with a much lower loss of the theoretically achievable lumen diameter than that of 32% reported for PTCA.[41]

Quantitative Angiographic Follow-up

Restenosis after PTCA is regarded as a local vascular wall response to the injury caused by balloon dilation.[14] A new potentially relevant factor related to the presence of the stent wires in the vessel wall is added after coronary stenting.[22] This factor may constitute a continuous stimulus for intimal hyperplasia affecting both its rate and its duration.[23] The vessel wall response to stent placement has been characterized by variable degree of inflammatory reaction, as well.[21,23,42] Animal studies have shown that the thickness of the intimal layer covering the stented vessel segments peaks at 4–8 weeks after stent deployment.[20,39,43] Whereas luminal changes had already ceased 2 weeks after balloon injury, they continued to show progression even after 3 months from stent implantation.[23]

Clinical studies have demonstrated that restenosis occurs in 20–56% of the patients managed with intracoronary stent placement.[19,44–49] The incidence of this event appears to be influenced by the indication for stenting.[19,49] The restenosis rate in the present study is similar to that of 33% found by Haude et al[48] and lower than that of 41% and 56% reported by Roubin et al[46] and Fajadet et al,[49] respectively, in patients who received stents in circumstances similar to ours. Patient characteristics may have played a role in this difference. The proportion of patients with multiple stents and of those with the stent placed in the left anterior descending coronary artery or a bypass graft is relatively low in our study population. Patients with a single stent in the study with highest incidence of restenosis had a recurrence rate of 33%, which is not different from ours.[49] Herrmann et al[19] reported recently on the outcome of 56 patients who received emergency Palmaz-Schatz stenting after failed PTCA caused by suboptimal result, threatened closure, or acute occlusion. The overall restenosis rate was 23%, being higher (43%) in the subgroup of patients with acute coronary occlusion as an indication for stent implantation.[19] A tendency for a higher restenosis rate was also present in our 10 patients whose dilated vessel was acutely occluded before stent placement.

Eighty-four of a total of 430 patients with Palmaz-Schatz stents who had two angiographies performed approximately at 6 and 12 months showed only a minimal increase (2.4%) in the number of restenosis cases.[47] Because of the nonprospective nature of the study, these data cannot serve as a typical example of the timing of restenosis after stent placement. Our serial angiographic follow-up study indicated a restenosis rate of 22.0%, 31.9%, and 33.2% at 3, 6, and 12 months, respectively, in 82 patients with emergency coronary stenting. Coronary luminal changes occurred mostly in the first 3 months but were not limited to this period. Relevant reduction in minimal luminal diameter also was seen in the second trimester, leading to an important increase in the number of restenosis cases. Notably, the new restenosis cases revealed at 6 months only already had a larger decrease of the lumen at 3 months than the patients remaining free of restenosis. This finding indicates that patients with significant lumen changes at an early angiographic control after stenting (<3 months), although not falling in the category of restenosis, should be carefully followed up because of their increased risk for developing it during the next few months. The 6-month course of luminal changes in our patients appears to be similar to that reported by Kimura et al.[36] Further changes in coronary lumen dimensions were very rare after 6 months. Only one patient was added to the restenosis cases, and no need for new recanalization interventions emerged after this period. Patients with restenosis who did not need repeat PTCA at 6 months showed no progression of coronary stenosis afterward. These findings suggest that the 6-month period represents the time limit after which no relevant progression in coronary lumen narrowing occurs both for patients with and those without restenosis after coronary stenting. Moreover, the time course of coronary luminal changes and restenosis after emergency coronary stent implantation as emerged from this study is not different from that found in patients after conventional PTCA.[15,16]

Our data are derived from a particular group of patients who developed large occlusive coronary dissections during PTCA. Findings of experimental studies suggest that restenosis depends on the degree of vessel injury.[50] Therefore, the presence of large dissections may have affected not only the overall restenosis rate but its temporal course in such a way that our reported findings could not be reliably extrapolated for other indications of coronary stent placement.

Conclusions

The results of this study suggest that both the overall incidence and the time course of restenosis after emergency coronary stenting resemble those seen after conventional PTCA. Stent implantation appeared to prevent most of the elastic recoil occurring after PTCA alone without succeeding in its complete elimination. After a peak at 3 months, the luminal changes were gradually attenuated and the coronary lumen remained almost stable in the period after the first 6 months. If not otherwise indicated, a 6-month angiographic control would reveal the vast majority of changes expected to occur after coronary stent placement.

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Rapamycin Resistance Tied to Defective Regulation of p27^{Kip1}

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The potent antiproliferative activity of the macrolide antibiotic rapamycin is known to involve binding of the drug to its cytosolic receptor, FKBP12, and subsequent interaction with targets of rapamycin, resulting in inhibition of p70 S6 kinase (p70^{S6K}). However, the downstream events that lead to inhibition of cell cycle progression remain to be elucidated. The antiproliferative effects of rapamycin are associated with prevention of mitogen-induced downregulation of the cyclin-dependent kinase inhibitor p27^{Kip1}, suggesting that the latter may play an important role in the growth pathway targeted by rapamycin. Murine BC3H1 cells, selected for resistance to growth inhibition by rapamycin, exhibited an intact p70^{S6K} pathway but had abnormally low p27 levels that were no longer responsive to mitogens or rapamycin. Fibroblasts and T lymphocytes from mice with a targeted disruption of the p27^{Kip1} gene had impaired growth-inhibitory responses to rapamycin. These results suggest that the ability to regulate p27^{Kip1} levels is important for rapamycin to exert its antiproliferative effects.

Rapamycin inhibits the mitogen-stimulated serine/threonine kinase p70^{S6K} (27, 41) and prevents cyclin-dependent kinase (CDK) activation, retinoblastoma protein (Rb) phosphorylation, and G_1 progression (1, 6, 10, 19, 22, 33, 35). Regulation of CDKs, which is crucial for orderly initiation and progression of the cell division cycle, involves CDK inhibitors among other mechanisms (34, 49). CDK inhibitors act stoichiometrically, and oscillations in their levels can have profound effects on cell proliferation (43). The CDK inhibitor p27^{Kip1} (39, 51) is present at high levels in quiescent cells and is downregulated by mitogenic stimulation (23, 37, 40, 43). Downregulation of p27 by mitogens can be blocked by rapamycin (37). The requirement of p27 for the antimitogenic response to rapamycin remains to be established.

Rapamycin acts by binding to the cytosolic protein FKBP12 (46), and this complex in turn binds to and inhibits FRAP (4). Activation of p70^{S6K} requires an additional input that is provided by FRAP (also known as RAFT) (3, 45), a mammalian kinase homologous to the yeast target of rapamycin (TOR) molecules (5, 19, 26). FRAP itself is not a mitogen-activated p70^{S6K} kinase but is required to render p70^{S6K} susceptible to activation by mitogenic signals (4, 7). As a result, rapamycin selectively prevents mitogen-induced p70^{S6K} activation without disrupting other known pathways (8, 25).

p27 levels are increased in response to mitogen deprivation, cell-cell contact, or addition of the antimitogen transforming growth factor • (39, 43, 50) and during myeloid cell differentiation (31) and neurogenesis (29). The high levels of p27 in quiescent macrophages, fibroblasts, or T lymphocytes are decreased in response to the mitogens colony-stimulating factor 1, serum, and interleukin-2 (IL-2), respectively (23, 37). Although p27 can be regulated at the mRNA level (31), its downregulation by mitogens occurs posttranscriptionally and involves ubiquitin-dependent degradation (38).

Inhibition of mitogen-stimulated downregulation of p27 by rapamycin raises the possibility that the regulation of p27 levels is critical for the antiproliferative activity of rapamycin. To address this question, we have taken several complementary approaches. We established rapamycin-resistant murine BC3H1 cells by culturing in the presence of the drug without any induced mutagenesis. Multiple independent rapamycin-resistant (RR) cell lines had intact p70^{S6K} regulatory responses but exhibited constitutively low p27 levels even after mitogen deprivation. Rapamycin no longer increased the p27 levels in these cells. Moreover, p27 null fibroblasts and T lymphocytes derived from p27$^{-/-}$ mice exhibited a significant resistance to growth inhibition by rapamycin. These results argue that the ability to block p27 downregulation contributes to the growth-inhibitory action of rapamycin.

MATERIALS AND METHODS

Establishment of RR cells. BC3H1 cells were grown in Dulbecco's modified essential medium (DMEM) with the addition of 20% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 • g/ml). Medium was changed every 48 to 72 h; rapamycin was added directly to the culture medium (at final concentrations ranging from 0.1 to 1 • M). Cells were cultured for multiple passages over a 6-month period in the presence of high concentrations of rapamycin (0.1 to 1 • M) followed by dilutional cloning. Mv1Lu cells were from the American Type Culture Collection (CCL64). The rapamycin-resistant cell line RR-3 has been deposited in the American Type Culture Collection, where it is available on request.

DNA synthesis and cell proliferation assays. DNA synthesis in BC3H1 cells was determined by measuring the incorporation of [^{3}H]thymidine into DNA. Microcultures (• 5,000 cells) were established in flat-bottom 96-well microtiter plates in the presence of various concentrations of rapamycin. After 48 h, cells were pulsed with [^{3}H]thymidine and harvested 16 to 20 h later. ^{3}H incorporation was measured in a liquid scintillation counter.

p27 immunoblot assay. Exponentially growing cells (BC3H1 or Mv1Lu) were cultured in medium containing 0.5 or 20% FBS in the presence of 20 nM rapamycin, 50 • M MG101, or 100 nM wortmannin for 24 h (Mv1Lu cells) or 16 h (BC3H1 cells) as indicated below. The cells were washed with phosphate-buffered saline (PBS) and lysed by scraping into hypotonic buffer (20 mM N-2-hydroxyethylpiperazine-N•-2-ethanesulfonic acid [HEPES]-KOH [pH 7.5], 5 mM KCl, 0.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], leupeptin [10 • g/ml], antipain [10 • g/ml], benzamidine hydrochloride [100 • g/ml], aprotinin [50 • g/ml], soybean trypsin inhibitor [100 • g/ml], pepstatin [10 • g/ml] with sonication. Protein concentration in the lysates were measured using the Bradford reagent (Bio-Rad), with bovine serum albumin as a standard. Cell lysates containing 50 • g of total protein were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (12% gel) and Western blotting (immunoblotting) using a polyclonal anti-p27^{Kip1} antibody (43). The filters were washed

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and incubated with the secondary antibody conjugated to peroxidase for 20 min, and p27 was detected by chemiluminescence using the Amersham ECL system.

Rb immunoblot assay. Exponentially growing cells were plated at approximately 30% confluence. After 24 h, plates were washed twice with PBS and placed in low-serum medium (DMEM plus 0.5% FBS) for 72 h. Plates were then stimulated with 20% FBS and treated with either no drug (control) or 100 nM rapamycin. At the indicated time points, plates were washed with ice-cold PBS and lysates were prepared by scraping cells into lysis buffer (50 mM Tris-HCl [pH 8.0], 120 mM NaCl, 1 mM EDTA, 0.1 mM NaF, 0.2 mM Na_3VO_4, 10 mM • -glycerophosphate, 1 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride, aprotinin [1 • g/ml], leupeptin [1 • g/ml], soybean trypsin inhibitor [10 • g/ml], 0.5% Nonidet P-40). Protein concentration in the lysates were measured by using the Bradford reagent (Bio-Rad), with bovine serum albumin as a standard. Cell lysates containing 50 • g of total protein were subjected to SDS-PAGE (7% gel). Filters were blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% dry milk for 1 h, followed by incubation overnight with anti-pRb antibody (1/1,000 dilution) at 4•C. The filters were washed with PBS-T and then incubated with the secondary antibody conjugated to peroxidase for 1 h. Detection was with the ECL system.

FKBP12, RAFT/FRAP, and p27 reverse transcription-PCR assays. FKBP12, FRAP, and p27 cDNAs were isolated from parental and rapamycin-resistant BC3H1 cells with reverse transcription of mRNA as previously described (21). Primers were synthesized based on the sequence of the murine FKBP12 5• and 3• untranslated regions: sense, 5•-GCCACCCGCGTCCTTTTCC-3•; and antisense, 5•-GGCAGATCCACGTGCAGAGC-3•. Degenerate oligonucleotide primers were prepared based on the reported protein sequence for FRAP/RAFT (45): sense, 5•-AA(A/G)AA(C/T)ATGTG(C/T)GA(G/A)CA-3•; and antisense, 5•-CACCA(T/C)TC(T/C)TGiGC(T/C)TCCAT-3•. Primers were synthesized based on the sequence of the murine p27 (39); sense, 5•-CTCCATCCGTGGC GTTT-3•; and antisense, 5•-CATTAACCCACCG-3•. PCR was performed for 30 cycles of 94•C for 1 min, 48•C for 1 min, and 72•C for 30 s. Products were ligated into pBluescript and sequenced on both strands.

$p70^{S6K}$ phosphorylation and activity assays. Cells were incubated in medium containing 20% FBS in the presence or absence of 20 nM rapamycin for 45 min. Cells were washed with PBS and lysed at 4•C in buffer containing 10 mM K(PO_4) (pH 7.4), 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 50 mM • -glycerophosphate, 1 mM Na_3VO_5, 2 mM DTT, 2 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40. Cell lysates containing 50 • g of total protein were separated by SDS-PAGE (7.5% gel) and analyzed by immunoblotting using anti-$p70^{S6K}$ antibody C2 (9). Loading on Western blots was controlled by measuring the protein concentration, using the Bradford assay. For S6 kinase activity assays, cell lysates containing 1 mg of total protein were immunoprecipitated with anti-$p70^{S6K}$ antibody N2 (9). The kinase activity of the immune complexes was determined by using an S6 peptide as a substrate as instructed by the supplier (Upstate Biotechnology, Inc.). Kinase activity was quantitated as the counts per minute of ^{32}P incorporated into the S6 peptide.

Determination of p27 half-life. Cells cultured under the indicated conditions for 24 h were placed in methionine-free medium for 30 min and then labeled with 200 • Ci of [^{35}S]methionine per ml for 40 min. Cells were then placed in full medium to chase the metabolic label and harvested at the indicated time points for lysis with hypotonic buffer. The lysates were immunoprecipitated with anti-p27 antibody, and the precipitates were analyzed by SDS-PAGE (12% gel) and with a PhosphorImager. The radioactive signal associated with the p27 band was quantitated by using ImageQuant software (Molecular Dynamics).

DNA and nuclear fragmentation assays. Exponentially growing cells were incubated in medium containing 0.5 or 20% FBS for 24 h. For nuclear staining assays, cells that had spontaneously detached from the dishes and attached cells released by trypsinization were collected washed with PBS and pooled. For nuclear staining assays (2), the cell suspension was fixed with 3% paraformaldehyde for 10 min at room temperature, stained with DNA-binding fluorochrome bisbenzimide (16 • g/ml; Hoechst 33085) in 1% paraformaldehyde for 15 min, spread on a coverslip, and examined under the microscope. At least 600 cells were counted to determine the proportion showing fragmented or condensed nuclei. For DNA fragmentation assays (15), cells were lysed in a solution containing 5 mM Tris-Cl (pH 8.0), 5% glycerol, 0.05% bromophenol blue, and 5 mg of RNase A per ml. The sample was loaded onto a discontinuous agarose gel with the part above the sample wells containing 1% agarose, 2% SDS, and 64 • g of proteinase K per ml and the part below the wells being 2% agarose. Gels were run at 60 mV for 14 h at room temperature. DNA was visualized by ethidium bromide staining.

Generation and analysis of p27 null mouse cells. Mice homozygous for a targeted disruption of the $p27^{Kip1}$ gene were obtained as described elsewhere (24). Embryonic primary fibroblasts were prepared from mouse embryos 13.5 days postcoitus as previously described (44). The fibroblasts were cultured in DMEM supplemented with 10% FBS. Exponentially growing fibroblasts (passage 4) were used in all experiments. $p70^{S6K}$ assays were done as described above. [^{125}I]iododeoxyuridine incorporation into DNA was determined in sparse cultures of wild-type and $p27^{-/-}$ mouse embryo fibroblasts after 24 h of incubation in the presence of the indicated concentrations of rapamycin. Cells were pulsed with [^{125}I]iododeoxyuridine and harvested 4 h later. ^{125}I incorporation was measured in a gamma counter.

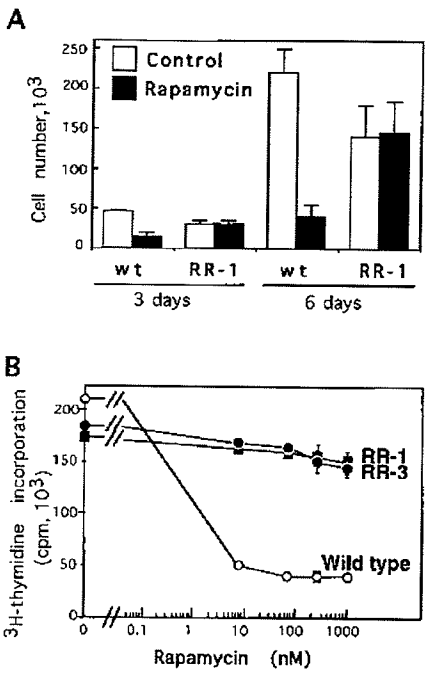

FIG. 1. Growth characteristics of RR BC3H1 cells. (A) Rapamycin inhibited cell growth in parental BC3H1 cells but not in RR cells. Cell cultures (10^4 cells each) were plated on day 0 in growth medium alone or containing 1 • M rapamycin. Cells counts were determined at the indicated times. Data are the averages of triplicate samples • standard deviations. wt, wild type. (B) Rapamycin inhibited DNA synthesis in parental BC3H1 cells but not in two independent RR cell lines. Cells were cultured with various concentrations of rapamycin for 48 h, at which point 1 • Ci of [^{3}H]thymidine was added. Cells were harvested 20 h later, and the [^{3}H]thymidine incorporated into DNA was counted. Data are the averages of four determinations • standard deviations.

Splenic lymphocytes were obtained by gently pressing the spleen against the bottom of a culture dish with a bent syringe needle. Cells were collected and incubated for 5 min at room temperature in 0.15 M NH_4Cl–1 mM $KHCO_3$–0.1 mM EDTA (pH 7.2) to lyse erythrocytes. Cells were pelleted, washed, and resuspended in RPMI 1640 medium with 10% FBS, 2 mM L-glutamine, nonessential amino acids, 1 mM sodium pyruvate, and 50 • M 2-mercaptoethanol. Splenic T lymphocytes were activated in RPMI 1640 with 10% FBS, 3 • g of hamster anti-CD3 antibody (Pharmingen) per ml, 10 • g of goat anti-hamster immunoglobulin G antibody (Chappel) per ml, 100 U of IL-2 per ml, and different concentrations of rapamycin. At 48 h after activation, cells were labeled with 10 • Ci of [^{3}H]thymidine per ml for 4 h. The labeled cells were harvested, and the incorporated radioactivity was measured by liquid scintillation counting.

RESULTS

$p70^{S6K}$ regulation in RR cells. BC3H1 cells are a murine myogenic cell line (47) that is growth inhibited by nanomolar concentrations of rapamycin (22). Rapamycin, as low as 0.2 nM, blocks cell cycle progression in BC3H1 cells (22). Prolonged incubation of these cells with rapamycin resulted in the selection of two independent clones, RR-1 (selected in the presence of 1 • M rapamycin) and RR-3 (selected at 0.1 • M rapamycin), that are resistant to growth inhibition by rapamycin, as determined by cell counts and thymidine incorporation assays (Fig. 1). Proliferation of RR-1 and RR-3 cells was unaffected by as much as 1 • M rapamycin (Fig. 1B).

The effects of rapamycin on $p70^{S6K}$ activity were examined in exponentially growing cells that were either serum starved, cultured in 20% FBS, or cultured in 20% FBS plus rapamycin (20 nM). Immunoblot analyses of total cell lysates indicated that rapamycin induced a switch in $p70^{S6K}$ from the hyperphos-

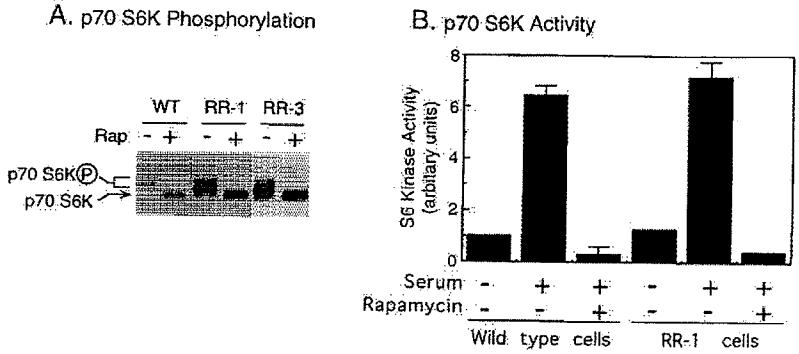

FIG. 2. Regulation of p70^{S6K} is intact in cells resistant to growth inhibition by rapamycin. (A) Parental BC3H1 cells (wild type [WT]) or two RR BC3H1 cell lines (RR-1 and RR-3) were cultured with 20% FBS and received 20 nM rapamycin for 45 min or no additions. Cell lysates (50 • g of total protein) were analyzed by Western immunoblotting using an anti-p70^{S6K}. The phosphorylated (active) forms of p70^{S6K} are the slow-migrating forms (9). (B) Parental BC3H1 cells and RR-1 cells were placed in medium containing 0.5% FBS, 20% FBS alone, or 20% FBS with 20 nM rapamycin. Cell lysates (1 mg of total protein) were immunoprecipitated with anti-p70^{S6K} antibody, and the kinase activity of the immune complexes was assayed with S6 peptide as the substrate. Data are expressed as relative activity compared to the S6 kinase activity in untreated wild-type cells. Data are the averages of two determinations, and bars show the range of values.

phorylated, active state (slower-migrating bands) to the hypophosphorylated state (faster-migrating bands) in both parental and RR BC3H1 cells (Fig. 2A). Similarly, in parental and RR cells, both serum starvation and rapamycin inhibited p70^{S6K} activity to levels as low as 5% of those seen with serum stimulation (Fig. 2B). Compared to wild-type cells, the levels of p70^{S6K} were 3-fold higher in RR-1 cells and 1.5-fold higher in RR-3 cells, based on immunoblot analyses (Fig. 2A). Although there was more p70^{S6K} protein in the RR cells, the levels of the active form (the most phosphorylated or slowest-migrating band) were similar. To ensure that the activity assays were accurate despite the increased level of p70^{S6K} protein in the RR cells, we performed p70^{S6K} activity assays with various amounts of cell lysate and determined that under the conditions used, the assay was in the linear range, indicating that the increased amount of p70^{S6K} protein in the RR cells was not saturating (data not shown). These results indicate that the p70^{S6K} inhibitory pathway is intact in the RR cells. This pathway is thought to involve rapamycin binding to FKBP12 and binding of this complex to FRAP (4). Indeed, cloning and sequencing of the endogenous FKBP12 and part of FRAP (corresponding to the region that confers rapamycin resistance when mutated in the yeast homolog [26]) showed that they were both wild type in the RR-1 cells, and the levels of FKBP12 protein as determined by immunoblot were also normal in these cells (data not shown).

Deregulated p27 levels in RR cells. p27 protein levels were determined in exponentially growing cells subjected to serum withdrawal, serum stimulation, serum plus rapamycin, or serum plus MG101 (an inhibitor of the ubiquitin-dependent degradation pathway involved in mitogen-induced p27 degradation [38]). Equal amounts of cell lysates were immunoblotted with an anti-p27 antibody. The p27 levels after serum starvation were • 5-fold lower in the RR-1 and RR-3 cells than in the parental cells and were comparable to the p27 levels in serum-stimulated parental cells (Fig. 3A). Furthermore, p27 levels in the RR cells were no longer regulated: they were neither decreased in response to serum nor increased in response to rapamycin (Fig. 3A).

The low levels of p27 in the RR-1 cells were not due to constitutive activation of a ubiquitin-dependent degradation process, since MG101, which prevented the downregulation of p27 by serum in parental BC3H1 cells, failed to stimulate the accumulation of p27 in RR cells (Fig. 3A). The lack of ubiq-

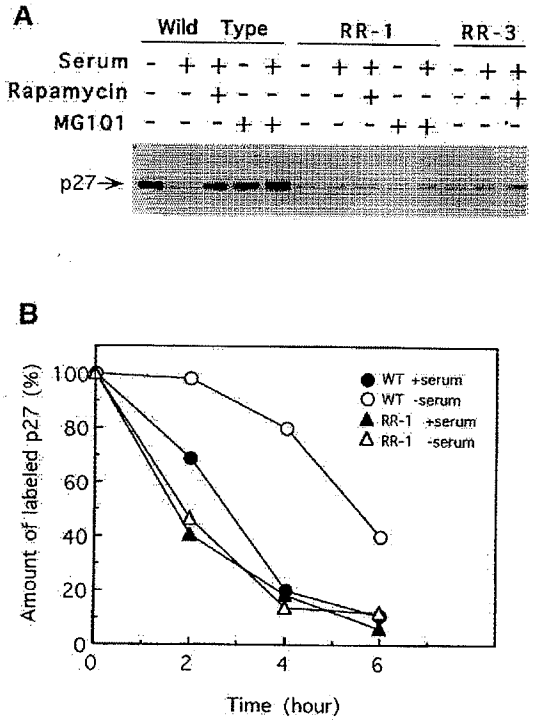

FIG. 3. p27 Levels are constitutively low in rapamycin-resistant cells. (A) Levels of p27 protein. Proliferating cell monolayers were placed in medium containing 0.5% FBS and received 20% FBS, 20 nM rapamycin, and/or 50 • M MG101 as indicated. After 24 h, cell lysates were prepared and aliquots (50 • g of total protein) were analyzed by Western immunoblotting using anti-p27^{Kip1} antibody C2. (B) Half-life of p27 in BC3H1 cells. Cell monolayers were incubated with 20 or 0.5% FBS for 24 h. Cells were then metabolically labeled with [^{35}S]methionine for 40 min, and the label was chased for the indicated times in complete medium. Cell lysates were immunoprecipitated with anti-p27 antibody N2, and the immune complexes were analyzed by SDS-PAGE and exposure in a PhosphorImager. The signal associated with the p27 bands was quantitated and is plotted as a percent of the signal at the start of the chase in each condition. The experiment was repeated twice with similar results in each case. WT, wild type.

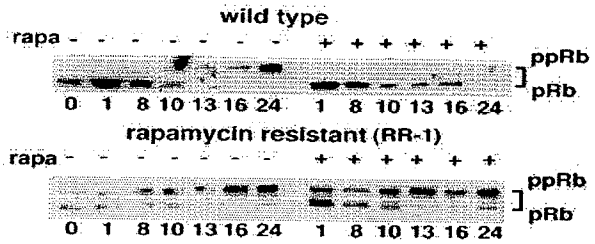

FIG. 4. Rb phosphorylation in RR cells is not affected by rapamycin and persists in mitogen-poor medium. Exponentially growing cells were placed in low-serum medium (DMEM plus 0.5% FBS) for 72 h. Cells were then stimulated with 20% FBS and treated with either no drug or 100 nM rapamycin (rapa). pRb was examined at the indicated time points (in hours) by Western immunoblotting using an anti-pRb antibody. The slow-migrating forms of pRb correspond to hyperphosphorylated forms (12).

uitin-mediated degradation of p27 in RR cells was not due to a generalized defect in the ubiquitin pathway, since MG101 did inhibit the degradation of cyclin B1 in both BC3H1 and RR-1 cells (data not shown). The loss of p27 in serum-deprived RR-1 cells incubated with MG101 (Fig. 3A) was a reproducible finding that we presume is due to a nonspecific toxic effect of MG101 on these cells when serum starved. A specific effect of MG101 would be to increase p27 levels rather than decrease them.

The p27 mRNA levels in parental and RR cells were similar, as determined by Northern (RNA) analysis (data not shown). However, the half-life of p27 in the RR cells was markedly decreased, as determined by immunoprecipitation and quantitation of labeled p27 from pulse-chase metabolically labeled cells (Fig. 3B). The half-life of p27 in RR1 cells was less than 2 h either in the presence or in the absence of serum, which was similar to the half-life of p27 in serum-stimulated parental cells and significantly less than the half-life (• 5 h) in serum-starved parental cells. Therefore, the low levels of p27 in the RR cells are due to a high rate of ubiquitin-independent degradation. Sequencing of the p27 mRNA from RR-1 cells showed that it was wild type (data not shown).

Rb phosphorylation and apoptosis in serum-starved RR cells. In various cell types, the decrease in p27 levels upon addition of mitogens helps activate CDKs, leading to Rb hyperphosphorylation and cell cycle progression (23, 37). Since the p27 levels in our RR cells were low already in the absence of serum, it was of interest to determine whether this anomaly would result in mitogen-independent Rb phosphorylation. Indeed, immunoblot analysis showed the presence of hyperphosphorylated (slow-migrating) Rb in serum-starved RR-1 cells, whereas this form was absent in serum-starved parental BC3H1 cells (Fig. 4). Furthermore, the progressive accumulation of hyperphosphorylated Rb upon mitogenic stimulation of serum-starved cells was blocked by rapamycin in parental BC3H1 but not in RR-1 cells (Fig. 4), which is consistent with the fact that the latter cells were able to proliferate.

In the course of this work, we noticed that the RR cells remained in a proliferative state when placed in mitogen-poor medium, as determined by [^{3}H]thymidine or [^{125}I]iododeoxyuridine incorporation into DNA, but failed to accumulate because of continued detachment from the culture dish and death. In several instances, it has been shown that blocking or bypassing Rb function causes apoptosis when cells are placed in mitogen-poor medium (11, 42, 48, 52). These and other studies have suggested that conflicts between activation of the Rb pathway in the absence of concurrent activation of other mitogenic pathways can induce apoptosis. Thus, the presence of inactivated (hyperphosphorylated) Rb in mitogen-deprived RR-1 cells raised the possibility that cells in this population could be driven to apoptosis, rather than growth arrest, by serum deprivation. Confirming this possibility, we found that following 24 h of serum deprivation, the RR cells were detaching from the dishes and showed apoptotic features including a typical DNA fragmentation ladder on agarose gels (Fig. 5A), as well as chromatin condensation and nuclear fragmentation in 35 to 43% of the entire (attached plus floating) cell population (Fig. 5B). The attached cell population, which was the population used for the p27 and p70^{S6K} assays described above, showed little evidence of apoptosis (• 3% nuclear fragmentation) when analyzed separately from the floating population of cells.

Impaired rapamycin response in p27$^{-/-}$ fibroblasts and T lymphocytes. The finding that selection for cell resistance to growth inhibition by rapamycin yields clones with constitutively low and deregulated p27 levels is consistent with the possibility that p27 plays a critical role in the growth-inhibitory action of rapamycin. To directly test this hypothesis, we used p27 null cells derived from mice that have a targeted disruption of the p27 gene (24). Primary embryo fibroblasts from p27$^{-/-}$ animals showed a normal p70^{S6K} inhibition response to rapamycin. p70^{S6K} phosphorylation was inhibited half-maximally at 0.1 nM rapamycin and fully at 1 nM rapamycin (Fig. 6A), which is very similar to the effect in wild-type fibroblasts T lymphocytes and other cell types (data not shown) (27, 41). However, exponentially growing p27$^{-/-}$ fibroblasts had an impaired antiproliferative response to rapamycin (0.1 to 1 nM), as determined by the rate of [^{125}I]iododeoxyuridine incorporation into DNA. The extent of inhibition of DNA synthesis in p27$^{-/-}$ fibroblasts was only half that observed in p27$^{-/-}$ fibroblasts (Fig. 6B). Cell density did not affect the rapamycin response, since similar effects were observed with sparsely seeded and densely seeded cells (data not shown).

The impairment of antimitogenic responsiveness to rapamycin in p27 null cells was confirmed using splenic T lymphocytes (Fig. 6C). This cell type was of particular interest because one of the most apparent phenotypic traits observed in p27$^{-/-}$ animals is an enlarged thymus and spleen owing to an increase in the number of T cells and the proportion of cycling cells (16, 24, 36). The inhibitory effect of rapamycin was less extensive in p27$^{-/-}$ T cells than in p27$^{+/+}$ T cells. The sensitivity to growth inhibition by rapamycin was 15- to 30-fold lower in p27$^{-/-}$ cells (50% inhibitory dose • 0.6 to 1 nM rapamycin) than in p27$^{+/+}$ cells (50% inhibitory dose • 0.03 nM rapamycin) (Fig. 6C). These results suggest that rapamycin exerts antimitogenic effects through several mechanisms; one requires p27 and is activated by rapamycin concentrations in the 0.03 to 1 nM range, whereas the other(s) is p27 independent.

DISCUSSION

Rapamycin has been used as a molecular probe to dissect the mitogen-stimulated signaling pathways that control cell proliferation. The rapamycin-FKBP12 complex binds to FRAP/RAFT and inhibits p70^{S6K} activation. A hallmark of rapamycin-induced interference with mitogenic signals is the inhibition of CDKs and Rb phosphorylation. Moreover, the antiproliferative response to rapamycin is associated with an increase in the levels of the CDK inhibitor p27^{Kip1}. Therefore, it has been proposed that regulation of p27, possibly involving p70^{S6K}, is critical to the antiproliferative activity of rapamycin, although there is no evidence for a direct link between these molecules. In this present study, we provide evidence that

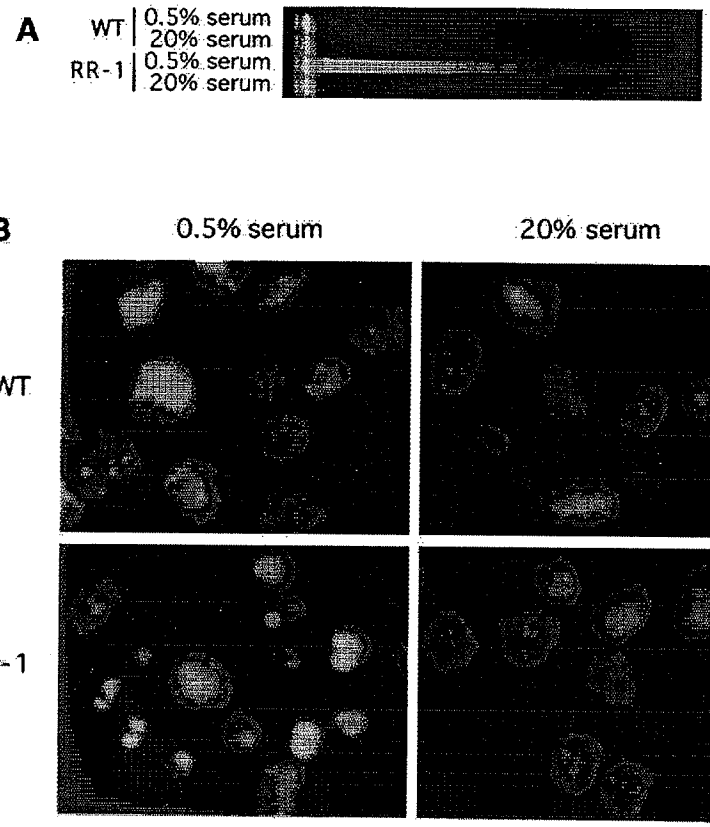

FIG. 5. Serum withdrawal induces apoptosis in the RR cells. Cells were cultured for 24 h in medium containing the indicated amounts of FBS. Cells that were attached to the culture dishes and cells that became spontaneously detached were pooled, and DNA fragmentation (A) and nuclear morphology (B) were examined as described in Materials and Methods. WT, wild type.

establishes the importance of p27 regulation in the antiproliferative activity of rapamycin.

Previous work showed that inhibition of Cdk2 by rapamycin is associated with increasing p27 levels in mitogenically stimulated T lymphocytes and human diploid fibroblasts (37). In the present study, independent BC3H1 cell lines that are resistant to inhibition of cell cycle regulators by rapamycin all exhibit the same abnormality: defective regulation of p27 levels. The constitutively low levels of p27 observed in the RR BC3H1 cells are explained by increased p27 degradation, as evidenced by a decrease in the half-life of the protein. Earlier studies demonstrated that changes in p27 levels quantitatively comparable to those observed between parental and RR BC3H1 cells can determine whether a cell will proliferate or arrest. For example, it has been shown that in an inducible p27 expression system, as little as a threefold increase in p27 levels can result in cell cycle arrest (43).

Rapamycin inhibits $p70^{S6K}$ activation (9, 27, 41), which is essential for G_1 progression in various cell types (28). However, in the RR cells, transition through G_1 to S does not depend on $p70^{S6K}$ activation, as these cells proliferate in the presence of rapamycin, even though $p70^{S6K}$ is inactivated. In the RR cells, constitutively low levels of p27 protein uncouple $p70^{S6K}$ from the antimitogenic effects of rapamycin. There are several possible explanations for this uncoupling. The best-known substrate of $p70^{S6K}$ is the 40S ribosomal protein S6 (8, 25), and S6 phosphorylation is thought to increase the translation rate of certain mRNAs required for G_1-phase progression. Moreover, in addition to ubiquitin-dependent degradation of p27, translational control also plays a role in regulating p27 levels during the cell cycle (20). It is conceivable, therefore, that under normal circumstances, $p70^{S6K}$ activates translation of an mRNA encoding a protein involved in p27 degradation, but that the abnormally short half-life of the p27 protein in the RR BC3H1 cells prevents significant changes in the levels of p27 protein. It is also possible that a protease is mutated in the RR cells such that it is constitutively activated resulting in low levels of p27 throughout the cell cycle.

In contrast to the mechanism of p27 downregulation by mitogens in parental BC3H1 cells, the short half-life of p27 in RR cells is not due to constitutive activation of a ubiquitin-dependent mechanism. This point is based on the observation that an inhibitor of ubiquitin-dependent degradation, MG101, prevents the serum-induced loss of p27 in parental cells but not the constitutive degradation of p27 in RR cells. The mechanism that leads to the short half-life of p27 in the RR BC3H1 cells remains to be determined. Characterization of RR mutants of YAC-1 murine T cells indicated that in these cells, the mutation was dominant (13, 14). This would be consistent with the constitutive activation of a protease that degraded p27.

A critical finding in the present study is that selection for cell resistance to the antimitogenic action of rapamycin yields clones with constitutively low levels of p27. The p27 levels in these cells are no longer affected by serum or rapamycin. Rapamycin resistance was established in BC3H1, a cell line that is normally growth inhibited by low concentrations of

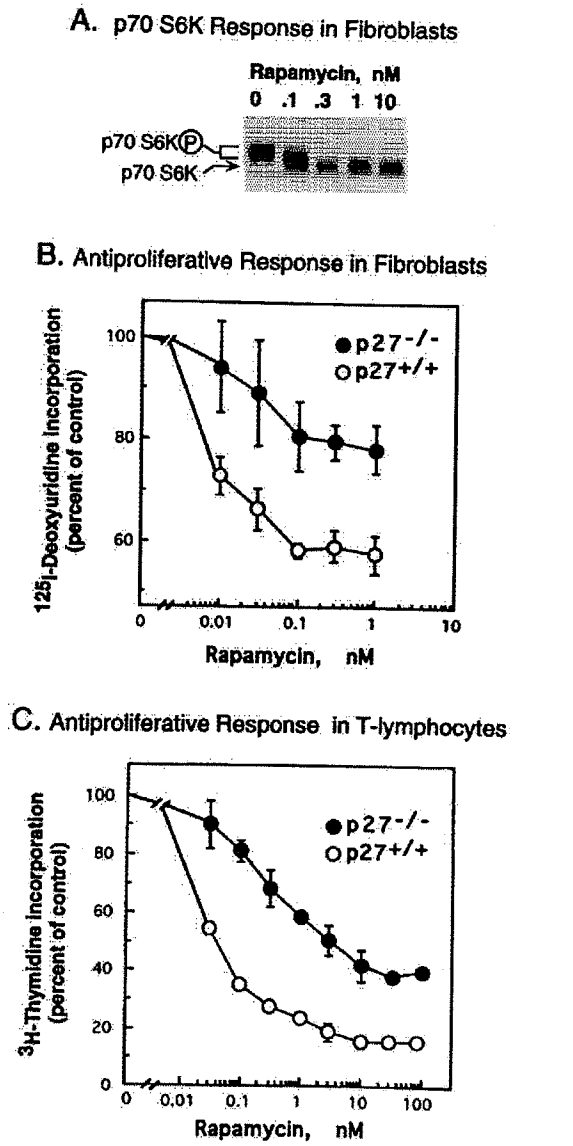

FIG. 6. Impaired growth-inhibitory response to rapamycin in p27 null cells. (A) The phosphorylation state of $p70^{S6K}$ was assessed by Western immunoblotting in extracts from $p27^{-/-}$ mouse primary embryo fibroblasts treated with the indicated concentrations of rapamycin for 45 min. (B) [^{125}I]iododeoxyuridine incorporation into DNA was determined in sparse cultures of wild-type and $p27^{-/-}$ mouse embryo fibroblasts after 24 h of incubation in the presence of the indicated concentrations of rapamycin. (C) Wild-type and $p27^{-/-}$ primary T lymphocytes were activated with anti-CD3 antibody and incubated with IL-2 and various concentrations of rapamycin for 48 h. [^{3}H]thymidine incorporation into DNA was then determined. In panels B and C, data are presented as the percent incorporation relative to controls that did not receive rapamycin and as the averages of triplicate determinations ± standard deviations. The experiments were repeated four times with similar results.

rapamycin in a manner very similar to that described for other rapamycin-sensitive cell types (22). The method chosen for establishing RR BC3H1 cells, selecting for cells that could grow in high concentrations of rapamycin, did not involve any mutagenesis protocols. The findings in the present study are based on parallel experiments using two entirely independent RR cultures. Importantly, $p70^{S6K}$ regulation by serum or rapamycin is intact in these cells. The fact that independent RR populations, selected without forced mutagenesis, both exhibited abnormal regulation of p27 supports the notion that p27 downregulation is a critical step in mitogenic stimulation of G_1 progression. To assess whether other cyclin inhibitors besides p27 were regulated abnormally in RR cells, we examined the levels of p15 and p21 in BC3H1, RR-1, and RR-3 cells. However, none of these CDK inhibitors are present at detectable levels in BC3H1 cells or in the RR cells. These data suggest that p27 is the major CDK inhibitor in BC3H1 cells, and since the levels of p15 and p21 were so low in BC3H1 and RR cells, we cannot comment on whether these other CDK inhibitors were also downregulated in RR cells.

The ability of rapamycin to inhibit $p70^{S6K}$ activation indicates that the rapamycin-FKBP12-FRAP pathway is intact in these resistant cells. Indeed, the levels of FKBP12 protein are normal in these cells, and we found no mutations in either FKBP12 or the region of FRAP that in the yeast homolog TOR2 contains a mutation that confers rapamycin resistance (26). Recently, additional mutations in TOR1 and TOR2 that confer rapamycin resistance by preventing rapamycin-FKBP12 binding to TOR1 and TOR2 have been described (32). Similar mutations in FRAP also block rapamycin-FKBP12 binding. However, the presence of these mutations in the RR BC3H1 cells is highly unlikely since the sensitivity of $p70^{S6K}$ activation to rapamycin is normal in these cells (4).

Two additional observations illustrate the functional significance of low p27 levels in these RR cells. First, when deprived of serum, the RR cells still contain hyperphosphorylated Rb and progress into S phase, both events are indicative of the presence of G_1 CDK activity. Second, the cells have a high propensity to undergo apoptosis under these conditions. Apoptosis in serum-free media has been found in systems in which the Rb pathway is activated without the concurrent activation of other mitogenic pathways. For example, blocking Rb action by overexpression of adenovirus E1A protein (11) or circumventing it by E2F overexpression (42, 48, 52) induces apoptosis when cells are placed in serum-free medium. This process appears to involve a p53-dependent checkpoint that triggers apoptosis in response to an imbalance of mitogenic signals (11, 42, 52). The high rate of apoptosis in our serum-deprived RR cells is therefore consistent with the presence of mitogen-independent Rb kinase activation.

The demonstration that cells from mice with a targeted disruption of the p27 gene are partially resistant to the antiproliferative effects of rapamycin provides further support for the hypothesis that rapamycin exerts its antimitogenic action in part by preventing mitogen-induced downregulation of p27. Although rapamycin blocks $p70^{S6K}$ activation in both primary cultures and BC3H1 cells, constitutively low levels or absence of p27 interferes with the antimitogenic effects of the drug. Moreover, the antimitogenic effect of rapamycin varies with the cell type, suggesting different degrees of dependence on rapamycin-sensitive mitogenic mechanisms. Serum-stimulated BC3H1 cells and IL-2-stimulated splenic T lymphocytes are profoundly growth inhibited by rapamycin, whereas primary mouse embryo fibroblasts are inhibited less extensively. More importantly, the results indicate that antimitogenic responses to rapamycin in both cell types are at least partially dependent on p27, since these responses are impaired in the $p27^{-/-}$ counterparts. p27 deficiency is associated with a decrease in both the amplitude and the sensitivity of the antiproliferative response to rapamycin. The decrease in sensitivity is most apparent in the $p27^{-/-}$ T lymphocytes, which require a 15- to 30-fold-higher concentration of rapamycin than do wild-type cells for a comparable level of growth inhibition. The maximal

difference in the rapamycin response of wild-type and p27 null cells is achieved at the low rapamycin concentration range that also achieves maximal inhibition of $p70^{S6K}$.

One interpretation of the results is that rapamycin may inhibit T-lymphocyte proliferation through at least two separate mechanisms, one that correlates with $p70^{S6K}$ inhibition and requires p27 and another that is p27 independent and occurs at higher rapamycin concentrations. Indeed, the partial sensitivity of the p27 null cells to rapamycin indicates that there is more than one pathway that determines rapamycin sensitivity. BC3H1 cells which are completely growth inhibited at the lowest concentrations of rapamycin (e.g., 10 pM) may have only the p27-dependent pathway, since rapamycin sensitivity is completely abrogated in the RR cells. However, it is also possible that p27-dependent and -independent mechanisms mediating rapamycin sensitivity are present in BC3H1 cells and are both lost in the RR cell lines.

Our observation of significant differences in rapamycin sensitivity between wild-type and p27 null fibroblasts and T cells is in contrast to the results of Nakayama et al. (36), who did not detect a difference in the effects of rapamycin on cell growth between wild-type and p27 null thymocytes. The discrepancy between our results and those of Nakayama et al. could be due to differences in cell culture and assay conditions. Nakayama et al. activated T cells by anti-CD3 plus anti-CD28 monoclonal antibodies and examined the effect of rapamycin on T-cell proliferation after 6 days in culture (36), whereas we activated T cells by anti-CD3 antibody plus its secondary antibody and our assays were done with T cells that had been in culture for only 2 days.

Kiyokawa et al. (24) have shown that there is no difference in apoptosis induced by either ionizing radiation or dexamethasone between wild-type and p27 null thymocytes. This finding is in agreement with our observation that RR cells can undergo apoptosis despite having low levels of p27.

The properties of the $p27^{-/-}$ cells described here support the conclusion that loss of p27 CDK-inhibitory function enhances the proliferative ability of the cell. Disruption of p27 in mice enhances postnatal growth owing to an increase in cell number in most organs (17, 24, 36). This phenotype is particularly apparent in the thymus and spleen, two tissues with relatively high p27 levels in normal mice. The increase in cell number is accompanied by an increase in the proportion of cycling cells, suggesting a propensity of p27 null cells to avoid quiescence or terminal differentiation. Our observation that cells from these animals have an impaired antimitogenic response to rapamycin supports the view that increased growth may result from an intrinsic hyperproliferative potential of the p27 null cells rather than the effect of a circulating somatotropic factor (24).

In conclusion, loss of p27 activity, whether as a result of cell selection in the presence of rapamycin or from targeted disruption of its gene, is associated with resistance to rapamycin. The level of resistance ranges from limited (e.g., in p27 null cells) to complete (e.g., in BC3H1 RR cells). Thus, rapamycin inhibits cell proliferation in part by opposing the mitogen-induced downregulation of p27. It also implies that p27 downregulation by serum or IL-2 is an important event in the mitogenic response to these agents. Thus, rapamycin represents a class of growth inhibitors that oppose the action of mitogens by blocking their signaling pathways, in the present case a pathway leading to downregulation of a CDK inhibitor. This mode of action distinguishes rapamycin from another class of antimitogens, represented by transforming growth factor •, which oppose the action of mitogens by regulating the expression of CDKs and their inhibitors (16, 18, 43) rather than by inhibiting mitogenic signaling pathways (30).

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The first two authors contributed equally to this work.

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CHAPTER

32 Restenosis

Marco A. Costa and Daniel I. Simon

Nature does nothing without purpose or uselessly.

—*Aristotle, 384-322 BC*

KEY POINTS

- Mechanisms of restenosis are different for balloon angioplasty, bare metal stents, and drug-eluting stents.
- Intimal hyperplasia with smooth muscle cell proliferation is the key mechanism of restenosis after implantation of bare metal stents.
- The biology of restenosis after drug-eluting stents varies, and tissue may have different cellular compositions (e.g., T lymphocytes) and fibrin deposition.
- The relative contribution of procedural and mechanical factors in the development of clinical restenosis is amplified after implantation of drug-eluting stents.
- Intimal hyperplasia after implantation of drug-eluting stents may appear as a homogeneous echolucent image on intravascular ultrasound and may be unnoticed during routine examination.
- The rates of restenosis and in-stent late lumen loss are lower with drug-eluting stents than with bare metal stents.
- The rates and patterns of restenosis are different for the various drug-eluting stent platforms.
- The best treatment for restenosis of drug-eluting stents remains to be determined, but emerging anti-restenosis strategies include catheter-based drug delivery systems and the use of biodegradable stents with or without combination drug therapy.

Restenosis is the arterial wall healing response to mechanical injury, which has plagued cardiologists since the introduction of balloon angioplasty by Grüntzig and collaborators.[1] This chapter describes the clinical features, mechanisms, and new facets of restenosis in contemporary percutaneous coronary intervention (PCI).

DEFINITIONS

Angiographic Restenosis

Angiographically detected obstruction of 50% diameter stenosis (DS) or more at the site of a previously treated coronary segment has been historically considered as representing restenosis.[2] This arbitrary cutoff point was founded on good scientific evidence; physiologic experimental studies demonstrated that when the arterial lumen diameter was reduced to 50% or less, coronary flow reserve became impeded.[3] For purposes of scientific studies, many definitions of angiographic restenosis have been used, although the classic binary definition based on the percentage of DS is the most widely accepted. Unfortunately, this measurement does not depict the degree of deterioration in lumen diameter and does not convey a measure of the vessel response to injury.[4,5] The use of the term percentage DS itself carries with it the assumption of normal-appearing reference segments, which is known to be an erroneous assumption given the diffuse aspect of coronary disease and neointimal proliferation. Binary restenosis assumes that a patient with 51% DS and another one with 49% DS have different intimal hyperplasia responses and outcomes.

In view of these considerations, clinical restenosis studies have been adopting a more comprehensive approach in reporting findings from both perspectives (categorical and continuous) to determine whether the agent under investigation had restraining or inhibitory effect and whether the ultimate clinical or angiographic outcome has been improved by the use of any new therapy. However, the more subtle facets of potent antiproliferative devices such as drug-eluting stents (DESs) challenge the validity of conventional angiographic parameters.[6-8]

Late loss is a continuous angiographic measure of lumen deterioration. Late loss is conventionally calculated by subtracting the minimal lumen diameter (MLD) value at follow-up from the postprocedural MLD. These computations are made irrespective of the locations of MLD measurements. Late loss has

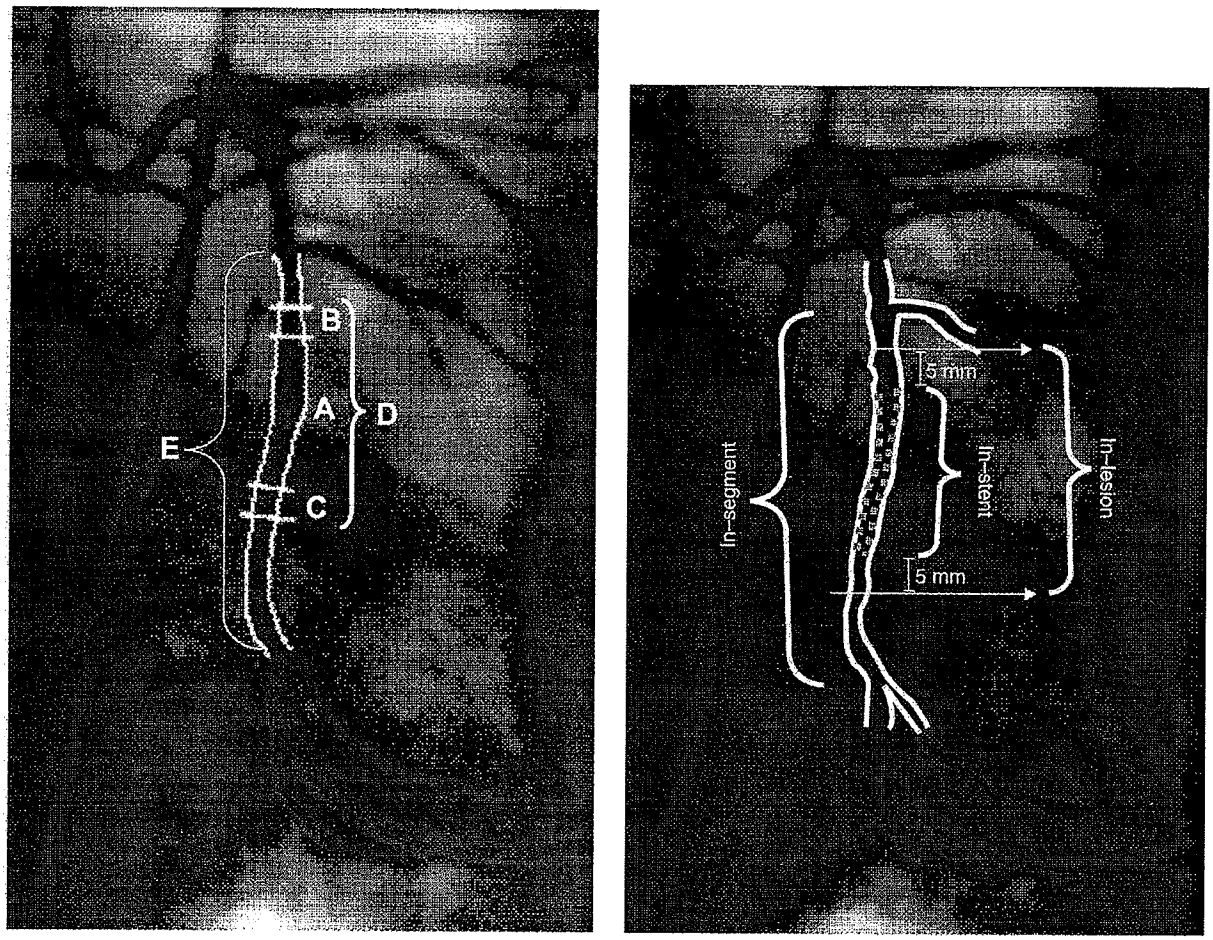

Figure 32-1. Coronary angiography and a schematic model illustrate a segmental approach to analyzing and reporting the effects of drug-eluting stents on coronary arteries. A, in-stent, which better describes the antiproliferative effect of the biologic agent; B, proximal edge (5 mm); C, distal edge (5 mm); and D, in-lesion sites determine potential paradoxical effects of low drug concentrations at traumatized stent edges (i.e. the edge effect); E, in-segment analysis is the ultimate determinant of angiographic success from a patient's perspective. (From Sousa JE, Serruys PW, Costa MA: New frontiers in cardiology: Drug-eluting stents. Part II. Circulation 2003;107:2383-2389.)

traditionally served as a major outcome measure in bare metal stent (BMS) trials and continues to play a similar role in the era of DESs.[9,10] It represents a surrogate marker of neointimal hyperplasia when measurements are performed within stented segments, because stents abolish the remodeling component of the restenotic process (discussed later).[4,11]

However, conventional late loss measurements may be methodologically flawed because of changes in the location of MLD between postprocedural and follow-up measurements.[8,12] As a result, late loss frequently is derived from measurements performed in different (unmatched) locations in the target segment. Sites with higher degrees of lumen deterioration and neointimal proliferation may be overlooked, potentially leading to inaccurate conclusions regarding the antiproliferative efficacy of a given device.

Angiographic restenosis parameters have been reported by means of in-lesion and in-stent analyses (Fig. 32-1). It is important to notice that in-lesion late loss will not necessarily be higher than in-stent late loss. These somewhat conflicting data, given that in-lesion analysis encompassed the stented segment and 5 mm distally and proximally in an attempt to depict edge restenosis, happen because of the MLD relocation phenomenon. In-lesion late loss also is affected by vascular remodeling or vessel spasm, and it cannot be used as a surrogate for intimal hyperplasia or to determine antiproliferative device efficacy.

Quantitative coronary angiography (QCA) has been used largely to determine restenosis parameters in the clinical context,[13] because visual assessment may lead to overestimation of the degree of narrowing in severe lesions and underestimation of the severity in mild or moderate lesions.[14] Digital systems permit online QCA in the catheterization laboratory, providing fast, easy, objective, and clinically relevant information for patient care.

Clinical Restenosis

Although angiography has been widely used as the guiding tool for coronary disease management, the clinician should also consider functional, invasive or

noninvasive assessment of the restenotic lesion before referring the patient to additional coronary revascularization. Grüntzig and coworkers observed that most clinical ischemic events related to vessel renarrowing occurred between 3 and 9 months after balloon angioplasty. This seminal observation illustrates the delay between the biologic process and symptomatic presentation of restenosis, which results in a 70% increase in the incidence of repeat revascularization between 6 and 12 months after the procedure.[15,16] Potent antiproliferative strategies may delay the biologic response to injury and extend the timeframe for developing clinical signs of restenosis. Intimal proliferation after brachytherapy seems to have a different time course.[17] Likewise, restenosis after DES implantation may be delayed, although a late catch-up restenotic phenomenon has not been observed in the initial studies.[18,19]

Restenosis may cause no symptoms in up to 50% of patients, although silent ischemia may be present and should be treated.[20] Exercise electrocardiographic testing has limited value for detecting silent restenotic lesions. Other noninvasive tests such as thallium scintigraphy and cardiac and stress echocardiography have been used to improve the sensitivity and specificity of noninvasive assessment of restenosis.[21] On the other end of the clinical spectrum, restenosis may manifest in the form of acute coronary syndrome in up to one third of patients, which challenges the notion that restenosis is benign.[22] This is of particular concern after left main PCI because of the risk of sudden cardiac death associated with early silent restenosis. As a result, routine 3- to 6-month angiographic follow-up should be considered for patients undergoing unprotected left main coronary artery PCI.

Target lesion revascularization (TLR), defined as any repeat percutaneous intervention of the treated coronary segment or bypass surgery of the target vessel, has been proposed as the most specific clinical restenosis end point among other clinical markers (i.e., death, myocardial infarction, symptom recurrence, or combined major adverse cardiac events).[4] Target vessel revascularization (TVR) expands the definition of TLR to include repeat percutaneous intervention of the target vessel, irrespective of the location of the stenosis within the treated segment.

In routine clinical practice, noninvasive assessment of recurrence of restenosis (i.e., symptomatic status and ischemia tests) appears to be an appropriate approach. This recommendation is based on a series of previous observations. Routine angiographic follow-up may have increased, albeit small, morbidity and mortality rates; asymptomatic patients with nonfunctional angiographic restenosis experience a benign course[23]; and the so-called occulostenotic reflex leads to a higher rate of repeat revascularization with no clear clinical benefit at 12 months after the initial intervention.[24] If repeat angiography is carried out without clear clinical evidence of ischemia, sensor-tipped guidewires for measurements of distal flow velocity or pressure may be useful for assessing the functional status of restenotic lesions in the catheterization laboratory and can assist physiologically based decision-making regarding the need for reintervention.[25]

MECHANISMS OF RESTENOSIS

The pathophysiology of restenosis is characterized by neointimal proliferation and negative vascular remodeling. The later contributes only to restenosis after PCI without stent implantation because the scaffold properties of stents abolish the remodeling process. It is nevertheless important to understand that the proliferative vascular response is enhanced by the persistent stimuli of rigid metallic struts in the vessel wall.

Neointimal hyperplasia was originally proposed to be a general wound-healing response.[26] Platelet aggregation, inflammatory cell infiltration, release of growth factors, medial smooth muscle cell (SMC) modulation and proliferation, proteoglycan deposition, and extracellular matrix remodeling were identified as the major milestones in the temporal sequence of this response. Initially, thrombosis was considered the main trigger of SMC proliferation after angioplasty. Platelet-derived growth factor (PDGF), which may also be secreted by endothelial cells and macrophages, is a potent promoter of SMC migration.[27] However, a more prominent effect of inflammation on SMC proliferation has been proposed.

Smooth Muscle Cell Proliferation and Restenosis

The SMC has long been implicated in the healing process after arterial injury[28] because of its ability to migrate, proliferate, and synthesize extracellular matrix (ECM) on stimulation.[29] After shifting from the contractile to the synthetic phenotype, SMCs may proliferate 24 hours to 2 to 3 months after vascular injury, returning to the contractile phenotype after this period. Adventitial myofibroblasts (α-actin–staining cells) also proliferate and migrate into the neointimal layer[30] and appear to play an important role in supplying the intima layer with proliferative cellular elements for new lesion formation. Through fracture of the internal elastic membrane, these cells migrate into the intima, where they may continue to proliferate and synthesize ECM, which will ultimately constitute the bulk of the restenotic lesion. ECM is composed of various collagen subtypes and proteoglycans[31] and constitutes the major component of the restenotic lesion; neointimal hyperplasia has been shown to be predominantly a low-cellular tissue.[32]

Cellular proliferative status in atherectomy specimens of restenotic lesions has been demonstrated by use of antibodies to proliferating cell nuclear antigen (PCNA), cyclin E, and cyclin-dependent kinase 2 (CDK2).[33] There is also evidence that cells of a monocyte-macrophage lineage (HAM-56$^+$) proliferate within human in-stent restenotic tissue.[34] The central

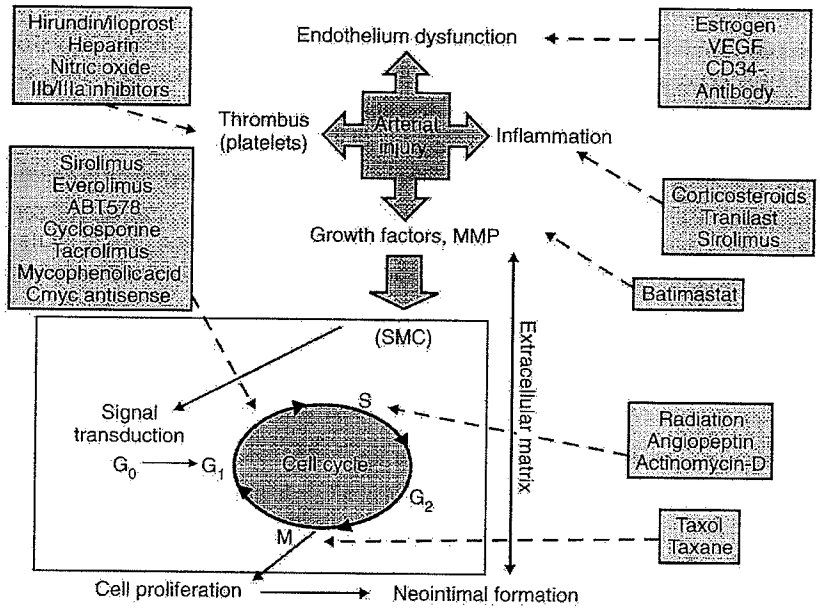

Figure 32-2. The leading processes of restenosis *(solid lines)* and corresponding inhibitory *(dashed lines)* effects of different anti-restenosis agents. MMP, matrix metalloproteinase; SMC, smooth muscle cell; VEGF, vascular endothelial growth factor. (From Sousa JE, Serruys PW, Costa MA: New frontiers in cardiology: Drug-eluting stents. Part I. Circulation 2003;107:2274-2279).

role of vascular cells in the restenotic process provided the basis for anti-restenosis pharmacologic strategies targeting cell cycle division early after stent implantation (Fig. 32-2).

Cell Cycle and Restenosis

The cell cycle is a common hub of the different phases of the restenosis process. The unprecedented clinical successes of recent anti-restenosis approaches targeting cellular division pathways illustrate its central role in the formation of neointimal hyperplasia. DES technologies deliver high concentrations of immunosuppressive or antitumor agents locally into the vessel wall. The specific molecular and cellular effects of these agents have been discussed in detail elsewhere.[6]

SMCs are quiescent and exhibit very low levels of proliferative activity. However, mechanical injury triggers the SMC progress through the G_1/S transition of the cell cycle.[35] The different phases of the cell cycle of eukaryotic cells are regulated by a series of protein complexes composed of cyclins (D, E, A, B), cyclin-dependent kinases (CDKs: CDK4, CDK2, CDC2 [p34]), and their cyclin-dependent inhibitors (CKIs: CDKN1B [p27 or KIP1], RPS6KB2 [p 70 or S6Kb], CDKN2A [INK4 or p16]). The function of CKIs is regulated by changes in their concentration and by their localization in the cell.[36] CDKN1B (i.e., p27 or KIP1) is downregulated after arterial injury when cell proliferation increases. CDKN1A (i.e., p21 or CIP1) is not observed in normal arteries, but it is upregulated along with CDKN1B in later phases of arterial healing response and is associated with a significant decline in cell proliferation and an increase in procollagen and transforming growth factor-beta (TGF-β) synthesis.[36] These findings suggest that CDKN1B and CDKN1A are endogenous regulators of G_1 transit in vascular SMC and inhibit cell proliferation after arterial injury. CDKN1B and CDKN1A bind and alter the activities of cyclin D–, cyclin E–, and cyclin A–dependent kinases (CDK2) in quiescent cells, leading to failure of G_1/S transition and cell cycle arrest.[37,38] Overexpression of CDKN1B results in cell cycle arrest in the G_1 phase. Conversely, inhibition of CDKN1B increases the number of cells in S phase.[39]

The level of CDKN1B is also regulated by constituents of the ECM. Mature collagen (i.e., polymerized type 1 collagen) suppresses RPS6KB2 and has been shown to increase the levels of CDKN1B, whereas monomeric collagen, which is present during degradation of ECM in the synthesis phase of restenosis, downregulates CDKN1B. The cell cycle also regulates SMC migration. These cells migrate during G_1 but not in later phases of the cell cycle.

Inflammation and Restenosis

The central role of autocrine or paracrine inflammatory mediators on SMC proliferation has been proposed.[41] Leukocyte recruitment and infiltration occur early at sites of vascular injury, where the lining endothelial cells have been denuded and platelets and fibrin have been deposited. The initial tethering and rolling of leukocytes on platelet P-selectin[42] are followed by their firm adhesion and transplatelet migration, processes that depend on leukocyte Mac-1[43] and platelet glycoprotein (GP) Ibα.[44] The precise cellular and molecular mechanisms of inflammation after arterial injury depend on the specific type of injury (i.e., stent versus balloon and mechanical versus atherogenesis). Experimental stent deployment in animal arteries causes a sustained elevation of MCP-1 after injury (≈14 days) compared with balloon-injured arteries (<24 hours).[45] Antibody-mediated blockade of CCR2, a primary leukocyte

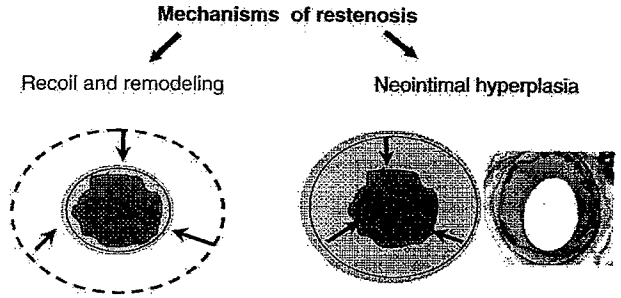

Figure 32-3. The two main mechanisms of restenosis, negative vessel remodeling and elastic recoil, are mainly observed after balloon angioplasty, whereas in-stent restenosis is mainly associated with intimal hyperplasia. In-stent restenosis histology is shown on the *right*.

receptor for MCP-1, markedly diminished neointimal thickening after stent-induced injury but not after balloon-induced injury in nonhuman primates.[46]

Investigators have pursued anti-restenosis strategies using systemic anti-inflammatory therapies, including liposome-encapsulated bisphosphonates,[47] prednisone,[48] anti-CD18 or anti-CCR2 blockade,[46] and the PPARG (PPAR-γ) activator rosiglitazone. Experimental observations support a causal relationship between inflammation and experimental restenosis. Antibody-mediated blockade[49] or selective absence of Mac-1[50] diminished leukocyte accumulation and limited neointimal thickening after experimental angioplasty or stent implantation. Corticosteroids reduce the influx of mononuclear cells, inhibit monocyte and macrophage function, and influence SMC proliferation.[51] However, clinical trials with systemic steroid therapy to prevent restenosis have shown disappointing results.[52]

Remodeling and Restenosis

The term *remodeling* has been applied largely to describe vascular shrinkage or enlargement. The definition proposed by Schwartz and colleagues,[53] in which remodeling is characterized in a continuous spectrum by any change in vascular dimension, may better describe this compensatory phenomenon. Studies using intravascular ultrasound provided the first evidence of the key role of negative remodeling (i.e., vessel shrinkage) on lumen deterioration after nonstented PCI.[54,55] Adventitial myofibroblasts, which are capable of collagen synthesis and tissue contraction as seen in wound healing,[56,57] may play an important role in negative vessel remodeling observed in restenosis after balloon angioplasty.

Remodeling is virtually absent after stenting as observed by volumetric intravascular ultrasound (IVUS) (Fig. 32-3).[58,59] The superior outcomes of bare metal stents compared with angioplasty result mainly from the scaffold property of these metallic prostheses, which prevents vessel shrinkage (i.e., elastic recoil and negative remodeling) despite inducing an enhanced neointimal hyperplasia response.

Specific Mechanisms of Bare Metal Stent Restenosis

The initial consequences immediately after stent placement are de-endothelialization, crush of the plaque (often with dissection into the tunica media and occasionally adventitia), and stretch of the entire artery.[60] A layer of platelets and fibrin are then deposited at the injured site. Activated platelets on the surface expressing adhesion molecules such as P-selectin attach to circulating leukocytes by means of platelet receptors, such as selectin P ligand (SELPLG, also called PSGL-1), and begin a process of rolling along the injured surface. Leukocytes then bind tightly to the surface through the leukocyte integrin class of adhesion molecules by direct attachment to platelet receptors such as GP Ibα and through cross-linking with fibrinogen to the GP IIb/IIIa receptor. Migration of leukocytes across the platelet-fibrin layer and diapedesis into the tissue is driven by chemical gradients of chemokines released from SMCs and resident macrophages.

The granulation or cellular proliferation phase occurs next. Growth factors are released from platelets, leukocytes, and SMCs that stimulate migration of SMCs from the media and adventitia into the intima layer. The resultant neointima consists of SMCs, extracellular matrix, and macrophages recruited over a several-week period. Cellular division takes place in this phase, which appears to be essential for the subsequent development of restenosis. Over a longer period, the artery enters a phase of remodeling involving ECM protein degradation and resynthesis (Fig. 32-4). Accompanying this phase is a shift to fewer cellular elements and greater production of ECM. In stented arteries, re-endothelialization of at least part of the injured vessel surface may occur.

Specific Mechanisms of Drug-Eluting Stent Restenosis

Our understanding on the mechanisms of restenosis after DES implantation is limited. However, the biology of restenosis is probably altered by the potent antiproliferative agents released from these devices. The variations in drug distribution, degree of injury, and tissue composition along the target vessel wall provide substrates for heterogeneous local vessel wall responses, which has been observed after balloon angioplasty.[61] We have found that the cellular composition of human DES restenotic tissue may vary from a T-lymphocyte infiltrate to tissues containing predominantly SMCs, similar to the pattern in bare metal stent restenosis. It remains largely unknown which clinical, anatomic, and local vascular factors govern the distinct vascular responses after DES, and it has yet to be elucidated whether these biologic features lead to different clinical consequences.

An echolucent intimal hyperplasia, called the *black hole*, has been identified in patients treated with DESs (Fig. 32-5).[62,63] The molecular mechanisms involved in the development of the black hole are not understood but likely represent an altered cellular response

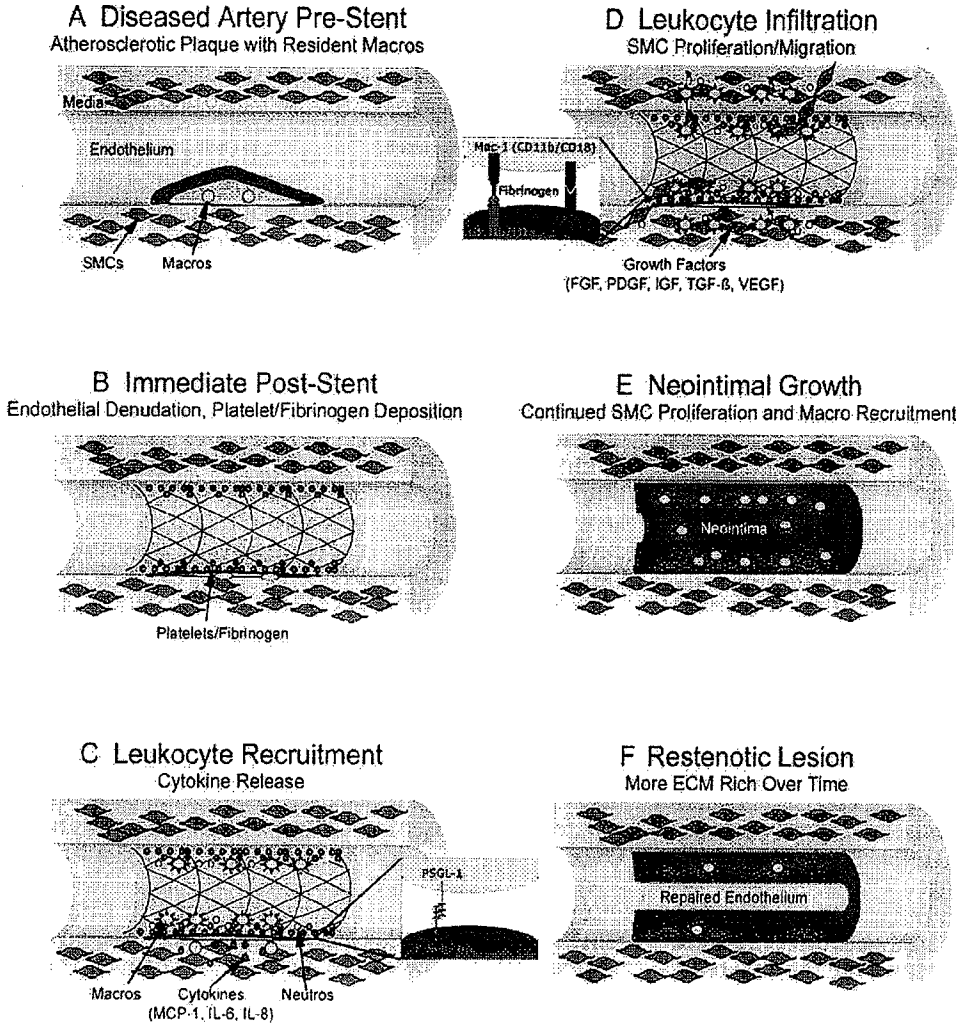

Figure 32-4. Schematic representation of an integrated cascade of restenosis. **A,** Atherosclerotic vessel before intervention. **B,** Immediate result of stent placement, with endothelial denudation and platelet and fibrinogen deposition. **C** and **D,** Leukocyte recruitment, infiltration, and smooth muscle cell (SMC) proliferation and migration in the days after injury. **E,** Neointimal thickening in the weeks after injury, with continued SMC proliferation and monocyte recruitment. **F,** Long-term (weeks to months) change from a predominantly cellular to a less cellular and more extracellular matrix-rich plaque. (From Welt FG, Rogers C: Inflammation and restenosis in the stent era. Arterioscler Thromb Vasc Biol 2002;22:1769-1776.)

to vascular injury. One possible explanation is the preservation of secretion properties of a few resident SMCs and inflammatory cells despite impairment of the cell cycle division. Regions of acellular, plasma-like collections were observed at 30 and 90 days after sirolimus-eluting stent implantation in porcine coronary arteries.[64] Experimental models have also shown increased fibrin deposition after DES implantation, which is considered a characteristic of delayed healing.[65] Fibrin accumulation was found at the site of a black hole in a patient who developed restenosis after DES treatment and was treated with atherectomy (Y. Oikawa, personal communication, 2006). Fibrin deposition was found in two cases of drug-eluting stent restenosis in our laboratory. Hypocellularity and the homogeneous characteristics of proteoglycans, fibrin, and plasma are likely responsible for the lack of ultrasound signal (i.e., echolucent). The black hole is a rare event and not always identified in DES restenosis tissue. This further supports the notion that the cellular mechanisms of restenosis after DES vary considerably.

Procedural Factors Associated with Restenosis

The "bigger is better" philosophy,[66] in which the lumen size obtained after PCI ultimately determines the occurrence of restenosis, has been largely accepted in the BMS era. The use of IVUS to guide optimal stent expansion, however, has not been shown to impact restenosis after BMS. Nevertheless, the Multicenter Ultrasound Stenting in Coronaries (MUSIC) IVUS study reported a 8.3% incidence of restenosis, which is likely the lowest rates ever reported in patients treated with BMS.[67] This study enrolled a much selected population and applied strict IVUS criteria of optimal stent deployment, which is difficult to achieve in routine practice.

Somewhat paradoxically, the potent antiproliferative effects of DESs exposed the mechanical- and procedural-related factors as major causes of restenosis. Neointimal hyperplasia is mostly abolished within the DES. As a result, the clinical signs of neointimal proliferation or negative remodeling in response to vessel trauma or untreated disease at the segments

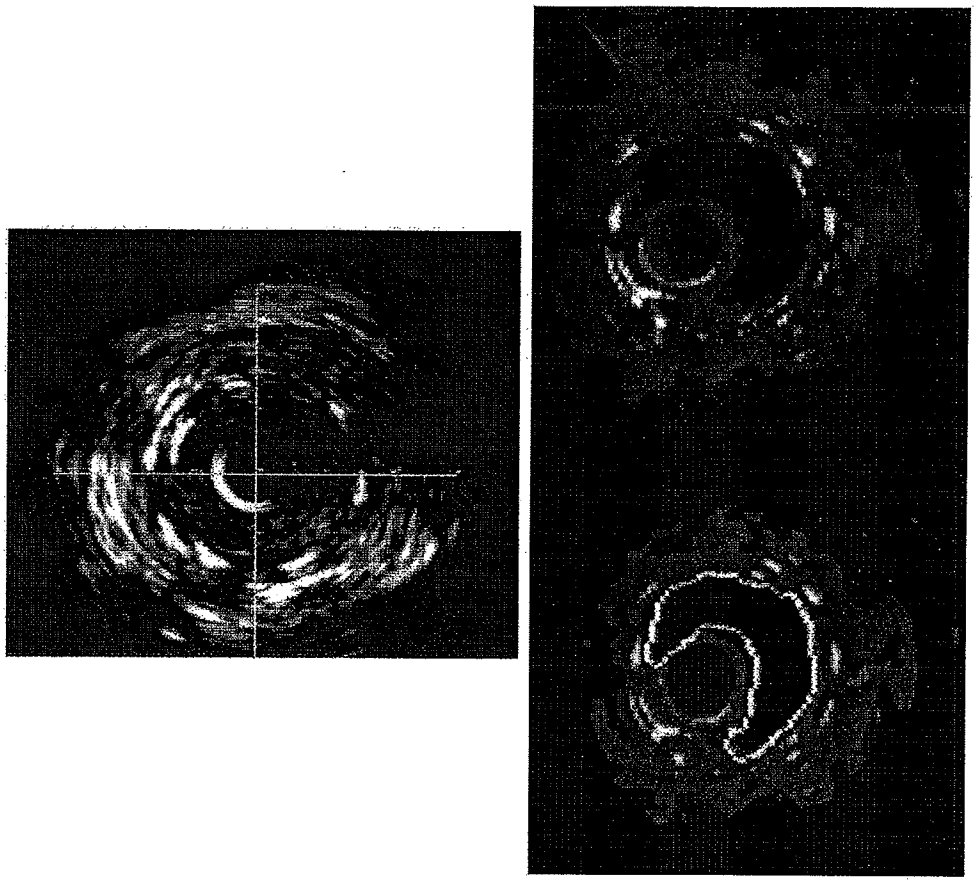

Figure 32-5. Example of a black hole *(left)* after implantation of a sirolimus-eluting stent, as detected by intravascular ultrasound (IVUS). The black hole has a homogeneous black appearance, but its differentiation from the lumen requires careful evaluation of the IVUS image. The typical IVUS appearance of intimal hyperplasia is shown after implantation of a bare metal stent *(right)*.

adjacent to the DES become amplified (Fig. 32-6). The deleterious clinical impact of suboptimal PCI techniques when applying antiproliferative devices was initially realized after the introduction of intracoronary radiation therapy.[68,69] At the time, the term *geographic miss* (GM) was used to describe a failure to fully cover the injured or diseased arterial segment with the radiation treatment source. There was a fourfold increase in the incidence of coronary-edge restenosis in patients with GM compared with those without GM undergoing intracoronary radiation therapy.[68,69] The STLLR study (i.e., prospective evaluation of the impact of stent deployment techniques on clinical outcomes of patients treated with the Cypher stent) was a large-scale ($N = 1567$, 43 participating institutions) study prospectively investigating sirolimus-eluting stent deployment technique and its relationship to clinical outcomes in the modern PCI era. This study reported a high incidence of GM as defined by the mismatching of lesion and injury vascular targets with subsequent sirolimus-eluting stent treatment deployment sites and provided the first scientific evidence of the negative impact of procedural related factors on clinical restenosis. Other mechanical-related failures that may trigger restenosis include stent underexpansion[70] and strut fracture.

Clinical Factors Linked to Restenosis

Identification of factors associated with a higher risk for restenosis may be useful in counseling patients about selecting PCI or other therapeutic strategies (i.e., clinical treatment or bypass surgery). Unfortunately, there have been inconsistencies in linking restenosis to baseline demographic and clinical characteristics. Diabetes mellitus has consistently been demonstrated to be an important clinical risk factor for restenosis after angioplasty and BMS implantation.[71] Some anatomic features have also been implicated with an increased likelihood of restenosis; saphenous vein graft disease, small vessel diameter, long lesions, and chronic total occlusion have been associated with a higher incidence of angiographic restenosis after BMS.[72-76] Although prior knowledge of the subset of patients at higher risk for restenosis may be useful for clinical decision-making, angiographic and IVUS studies have extensively demonstrated that the principal determinant of restenosis is the lumen size achieved at the end of the procedure.[77,78]

Although DES has drastically reduced angiographic and clinical restenosis across broad lesion and patient subsets, certain anatomic and clinical scenarios, such as patients with diabetes mellitus, restenotic lesions

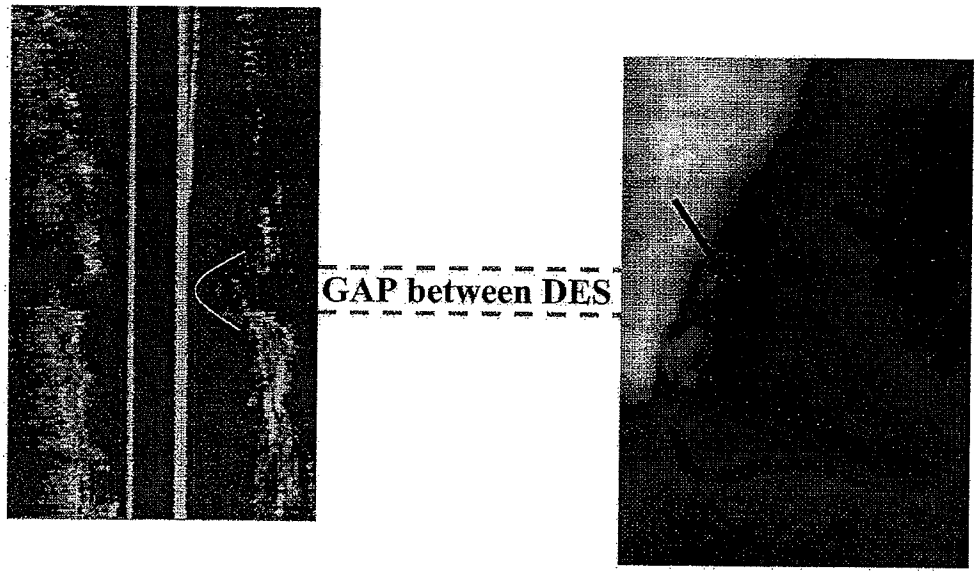

Figure 32-6. Longitudinal intravascular ultrasound (IVUS) image shows a focal intimal hyperplasia formation *(delineated)* in a gap between two sirolimus-eluting stents. Notice the lack of intimal hyperplasia within the stents. The corresponding angiographic illustration *(right)* shows restenosis *(arrow)*. Mechanical and procedural factors are key determinants of restenosis after implantation of drug-eluting stents (DESs).

after brachytherapy or DES implantation,[79] bypass graft disease,[80] and bifurcations, continue to be problematic.[81,82] Studies have reported a higher propensity for restenosis after implantation of paclitaxel-eluting stents than with sirolimus-eluting stents.[82-84]

INCIDENCE AND PATTERNS OF RESTENOSIS

The incidences of binary restenosis rate and late loss in key stent clinical trials are described in Table 32-1.[10,67,82,84-92] The differences in time of follow-up assessment, the percentage of patients with angiographic follow-up data, and the patient population should be considered when interpreting clinical trial restenosis data.

In-stent restenosis has been characterized angiographically as focal (≤10 mm long), diffuse (>10 mm), proliferative (>10 mm extending outside the stent), or total occlusion in patients treated with Palmaz-Schatz BMSs. This classification correlated with outcomes after reintervention and was largely adopted in the era of BMSs.[93] Focal restenosis occurred in 42% of patients, diffuse restenosis in 21%, proliferative restenosis in 30%, and total occlusion in 7% after Palmaz-Schatz stent implantation.[93,94] The mid-stent articulation, which provides less scaffolding support, was likely associated with the somewhat high frequency of focal restenosis in Palmaz-Schatz stents. The same classification has been used to evaluate restenosis after DES implantation, although modern stents do not have articulations and longer stents are used. Nevertheless, the pattern of in-stent restenosis has changed with DESs, and it appears to be specific for each type of device. Restenosis after implantation of sirolimus-eluting stents is mostly (>90%) focal and usually located at the stent edges,[95,96] whereas diffuse intimal proliferation or total occlusion accounts for approximately one half of the restenosis cases after using polymer-coated, paclitaxel-eluting stents.[97]

Assuming that intimal hyperplasia is almost completely blocked in nondiabetic patients with de novo, short (<20 mm) stenosis located in nonbifurcated native coronary arteries, restenosis is likely caused by technique-related failures. Restenosis would be mostly focal and observed at the stent edges or gaps between stents in these noncomplex cases (see Fig. 32-6). Biologic and mechanical failures likely contribute to more diffuse patterns of in-stent restenosis, seen mostly in more challenging clinical scenarios such as patients with in-stent BMSs, bypass graft disease, or diabetes mellitus. An exception to this rule is restenosis after bifurcation PCI, which should be classified separately because it is usually associated with focal stenosis at the ostium of the side branch.[98]

TREATMENT

Restenosis after balloon angioplasty has a relatively benign outcome after PCI. The 18% rate of repeat restenosis for patients treated with BMSs compares favorably with the data after treatment of de novo lesions (see Table 32-1).[99] Conversely, treatment of in-stent restenosis has been associated with high (>35%) rates of repeat TLR.[100] At 4 years of follow-up, the event-free survival rate was 69% after repeat BMS implantation and 64% after balloon angioplasty.[101]

Intracoronary radiation therapy was the sole therapeutic approach that proved clinically its efficacy for the treatment of BMS restenosis.[102] However, the treatment paradigm for in-stent restenosis changed recently, and DES implantation became the treatment of choice for restenosis of BMSs. Sirolimus-eluting stents and paclitaxel-eluting stents have been shown to be superior to brachytherapy for the treatment of BMS restenosis.[103-105] No data are available on the use of DESs for the treatment of postangioplasty restenosis.

Table 32-1. Summary of Clinical Data for Drug-Eluting Stents

Study	Randomized	Drug or Agent	Device Type	In-Stent Late Loss* (Follow-up Time)	In-Lesion Restenosis (Follow-up Time)
Pivotal Stent Trials					
STRESS (N = 410)	Yes	None	Palmaz-Schatz	0.74 mm (6 mo)†	31.6% (6 mo)
			Balloon angioplasty	0.38 mm (6 mo)†	42.1% (6 mo)
BENESTENT (N = 520)	Yes	None	Palmaz-Schatz	0.65 mm (6 mo)†	22% (6 mo)
			Balloon angioplasty	0.32 mm (6 mo)†	42% (6 mo)
MUSIC (N = 161)	No	None	IVUS-guided, Palmaz-Schatz	0.77 mm (6 mo)	8.3% (6 mo)
Pivotal DES Trials					
RAVEL (N = 238)	Yes	Sirolimus	BX Velocity	−0.01 mm (6 mo)	0% (6 mo)
			BX Velocity	0.8 mm (6 mo)	26.6% (6 mo)
SIRIUS (N = 1058)	Yes	Sirolimus	BX Velocity	0.17 mm (8 mo)	8.9% (9 mo)
	Yes	None	BX Velocity		
TAXUS II (N = 536)	Yes	Paclitaxel	NIR	0.31 mm (SR, 6 mo)	5.5% (6 mo)
				0.3 mm (MR, 6 mo)	8.6% (6 mo)
	Yes	None	NIR		
TAXUS IV (N = 1314)	Yes	Paclitaxel	Express 2	0.39 mm (SR, 9 mo)	7.9% (9 mo)
		None	Express 2	0.92 mm(9 mo)	26.6% (9 mo)
ENDEAVOR II (N = 1197)	Yes	Zotarolimus	Driver	0.61 mm (9 mo)	13.2% (9 mo)
		None	Driver	1.03 mm (9 mo)	35% (9 mo)
Head-to-Head Trials					
REALITY (N = 1386)	Yes	Sirolimus	BX Velocity	0.09 mm (8 mo)	9.6% (8 mo)
		Paclitaxel	Express 2	0.31 mm (8 mo)	11.1% (8 mo)
SIRTAX (N = 1012)	Yes	Sirolimus	BX Velocity	0.13 mm (9 mo)	6.7% (9 mo)
		Paclitaxel	Express 2	0.25 mm (9 mo)	11.9% (9 mo)
Diabetes Trials					
DIABETES-I (N = 160)	Yes	Sirolimus	BX Velocity	0.08 (9 mo)	7.7% (9 mo)
		None	BX Velocity	0.66 mm (9 mo)	33% (9 mo)
DIABETES-II (N = 80)	No	Paclitaxel	Express 2	0.42 mm (9 mo)	7.6% (9 mo)
ISAR-DIABETES (N = 250)	Yes	Sirolimus	BX Velocity	0.19 mm (6 mo)	6.9% (6 mo)
	Yes	Paclitaxel	Express 2	0.49 mm (6 mo)	16.5% (6 mo)

* Pivotal trials data provided for the active treatment groups only.
†In-lesion late loss is provided for bare metal stent studies because in-stent angiographic measurements were not applied. Drug-eluting stent figures reflect in-stent late loss.
BENESTENT, Belgian Netherlands Stent Study; DES, drug-eluting stent; DIABETES, Diabetes and Sirolimus-Eluting Stent Trial; ENDEAVOR II, Comparison of the Endeavor ABT-578 Drug Eluting Stent with a Bare Metal Stent for Coronary Revascularization; FR, fast release; ISAR, Intracoronary Stenting and Antithrombotic Regimen; IVUS, intravenous ultrasound; MUSIC, Multicenter Ultrasound Stenting in Coronaries Study; RAVEL, Randomized Study with the Sirolimus-Eluting Velocity Balloon Expandable Stent; REALITY, Prospective, Randomized Multicenter Head-to-Head Comparison of the Sirolimus-Eluting Stent (Cypher) and the Paclitaxel-Eluting Stent (Taxus); SIRIUS, Sirolimus-Eluting Bx-Velocity Balloon Expandable Stent in the Treatment of Patients with De Novo Native Coronary Artery Lesions; SIRTAX, Sirolimus-Eluting Stent Compared with Paclitaxel-Eluting Stent for Coronary Revascularization; SR, slow release; STRESS, Stent Restenosis Study; TAXUS, Taxus stent studies.

Newer approaches using balloon systems coated with hydrophobic agents such as paclitaxel have been tested in humans with promising results. A pilot study including a limited number of patients (N = 52) with BMS in-stent restenosis showed that recurrent restenosis rates were lower in patients treated with paclitaxel-coated balloon catheters compared with conventional balloon angioplasty (Fig. 32-7).[106] Larger studies comparing catheter-based versus stent-based delivery systems for the treatment of BMS restenosis are needed. Catheter-based strategies may also become an alternative treatment for DES restenosis in the future.

Modern interventional cardiologists must deal with a much less frequent, but still vexing, problem of restenosis after DES implantation, and the most appropriate treatment strategy has not been defined. Repeat restenosis rates up to 51% have been reported for repeat PCI of DES restenotic lesions. Outcomes appear to be associated with the pattern of the stenosis, with the highest repeat restenosis rates observed in patients with diffuse patterns.[107,108] Although the strategy of repeat DES implantation has been largely used, the safety of exposing the vessel wall to another potent antiproliferative therapy such as irradiation or a DES remains to be determined. The use of IVUS may be helpful in defining the mechanism associated with DES restenosis and should be considered by the physician. If mechanical failures are encountered, and the stenosis is discrete, properly sized balloon angioplasty may suffice. If the disease is mostly outside the stent (i.e., edge restenosis), another DES may be effective. A safe and effective strategy for the rare cases of diffuse or proliferative DES restenosis remains to be established.

FUTURE DIRECTIONS

There is room for improvement in the current DES delivery systems. Better flexibility and conformability are likely high on the wish list of most modern interventional cardiologists, who no longer fear

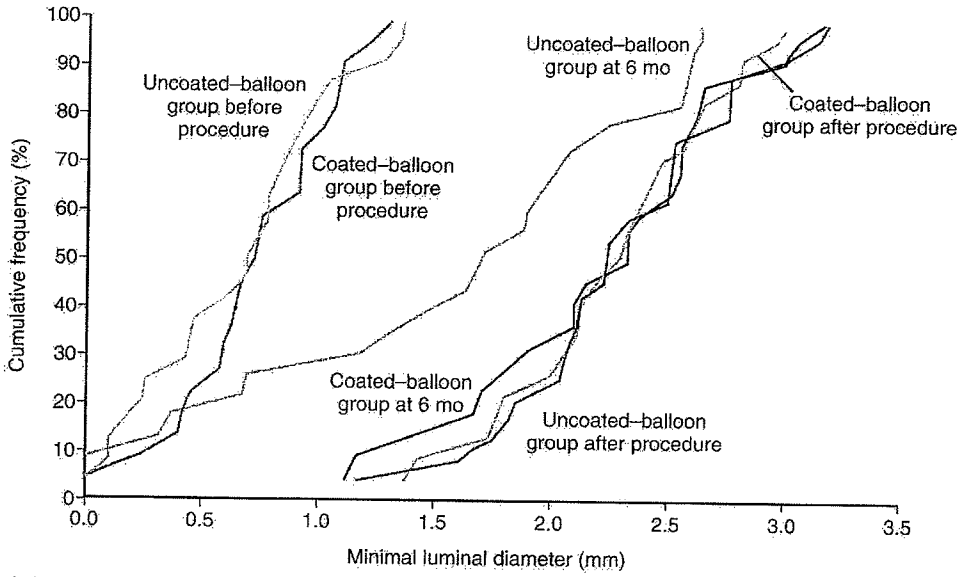

Figure 32-7. Cumulative frequency distribution of in-segment minimal luminal diameter on quantitative coronary angiography. Data are shown for the uncoated-balloon group and the coated-balloon group before the procedure, after the procedure, and at 6 months. (From Scheller B, Hehrlein C, Bocksch W, et al: Treatment of coronary in-stent restenosis with a paclitaxel-coated balloon catheter. N Engl J Med 2006;355:2113-2124. Copyright 2006 Massachusetts Medical Society. All rights reserved.)

restenosis but lack the appropriate tools to treat more complex disease.

Future anti-restenosis strategies will need to reconcile antiproliferative strategies with positive healing effects. Delayed endothelial dysfunction may persist for months after vascular injury and is likely more pronounced after DES implantation than with BMSs or balloon angioplasty.[109,110] Investigators have pursued diverse strategies, including pharmacologic combinations, tissue engineering, gene and stem cell therapies, and even procedural modifications (i.e., direct stenting) to limit endothelial injury and accelerate endothelial cell regeneration.[111-113]

Combination chemotherapy (i.e., use of multiple agents to optimize efficacy and limit toxicity) is the principal treatment strategy in oncology. Single-drug approaches have dominated DES programs, but there is emerging experimental evidence that combination anti-restenosis strategies may be effective in reducing neointimal thickening after stenting.[114] The use of antithrombotic agents associated with cell cycle inhibitors also may improve the safety of DES implantation.[115]

The catheter-based drug delivery approach has been resurrected. Local drug-delivery systems may provide high concentrations of antiproliferative drugs at the site of vascular injury without the undesirable persistence of a metallic prosthesis, which may remain prone to thrombosis if re-endothelialization does not occur. However, initial catheter-based local drug-delivery therapies have been unsuccessful in humans because of the rapid washout of the drug downstream into the coronary circulation and the potential flow- or pressure-mediated vessel wall injury.[116] The use of balloon-coating technology and hydrophobic drugs, which may minimize jet-induced injury and increase drug retention, produced satisfactory results in patients with in-stent restenosis (discussed earlier).[106] However, balloon-based PCI does not provide scaffold support, and vessels may be prone to dissection and abrupt closure. The predictability of the acute results of stent-based PCI is likely the main reason for the widespread acceptance of BMSs in the early days. A combination of balloon-based strategies and stents may play an important role in the future. Antithrombotic or prohealing agents may be used in conjunction with BMSs or DESs. Alternatively, the balloon may be coated with potent antiproliferative agents for early release, and the stents may carry healing drugs to trigger re-endothelialization.

Biodegradable DESs, which dissolve slowly after implantation, represents the ultimate stent technology. Theoretically, biodegradable or erodable stents provide initial scaffolding support to prevent vessel recoil and negative remodeling without the undesirable continuous vessel injury caused by a permanent, rigid foreign body. However, polymeric stents have yet to achieve the mechanical strength or surface properties of current stent technologies, and vessel toxicity remains a major limitation for polymeric biodegradable stents.[117] Biocorrosion of magnesium AE21 alloy containing 2% aluminum atoms and 1% rare earth elements (e.g., Ce, Pr, Nd) was well tolerated in pig coronary arteries,[118] although the vessel toxicity and hydrogen formation that may be associated with the corrosion of magnesium remains a concern.

SUMMARY

DESs represent a major breakthrough in the treatment of coronary disease and prevention of restenosis. However, restenosis has not been completely eradicated as initially thought, and the best treatment strategy for diffuse DES restenosis remains controversial. From better deliverability to biodegradable devices, DES platforms can improve considerably in the future. The current challenge of anti-restenosis strategies is to establish their long-term safety.

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